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BIOCHEMICAL PROPERTIES AND BIOACTIVITIES OF CARBON MONOXIDE-RELEASING MOLECULES (CO-RMS)

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THESIS SUBMITTED FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY IN THE UNIVERSITY OF LONDON

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Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science.

Jules Henri Poincaré (1854-1912)

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Abstract

Carbon monoxide (CO), synonymous of the "silent killer", is rapidly emerging as an important and versatile mediator of physiological processes. The study of CO has been hampered by the lack of a means to simulate its release biologically. Current means to replicate the effects of CO include, most notably, the use of CO gas and upregulation of haem oxygenase-1 (HO-1) to generate endogenous CO. Both are limited in their approach and offer only a partial solution. The recent discovery that certain transition metal carbonyls function as CO-releasing molecules (CO-RMs) in biological systems highlighted the potential of exploiting this and similar classes of compounds as a stratagem to deliver CO for research and therapeutic purposes. Initially a large portfolio of CO-RMs was investigated to determine their CO releasing capability. This thesis examines a number of aspects related to the characterisation of a core group of CO-RMs including: a) CORM-3, the prototypic water soluble transition metal carbonyl; b) CORM-A1, a water soluble CO-RM without a metal centre; c) CORM-319, an iron based water soluble CO-RM; and d) CORM-311, an ethanol soluble iron centred CO-RM. Specifically, the study will examine CO-RMs for their ability to: i) release CO; ii) suppress LPS-induced nitrite production; iii) promote toxicity; iv) induce haem oxygenase (HO) activity and HO-1 expression and v) modulate inducible nitric oxide synthase (iNOS) expression. These different aspects of CO-RM characterisation were addressed using biochemical, molecular biology and cell culture techniques. Further work was also carried out determining certain chemical aspects of each CO-RM including the decomposition rate and pH/temperature stability. The study into the CO release of the new CO-RMs emphasizes the versatile potential of the

metal carbonyl complexes and related compounds. This research on CO-RMs will help lay the foundations for a novel therapeutic agent based on the delivery of safe and controlled quantities of CO.

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List of Abbreviations

BVR	Biliverdin reductase
cGMP	Cyclic guanosine-5'-monophosphate
CO	Carbon monoxide
CO-RM	CO releasing molecule
EDRF	Endothelium derived relaxing factor
Fe(III)	Ferric iron
GSH	Glutathione
HbCO	Carboxyhaemoglobin
HO	Haem oxygenase
h	Hour
iNOS	Inducible NOS
LPS	Lipolysaccharide
MbCO	Carboxymyoglobin
Min	Minute
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2^-	Superoxide
OH^-	Hydroxyl radical
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
TNF	Tumour necrosis factor

1 Introduction

1.1 The Haem Oxygenase Pathway

Haem oxygenase (HO) is the rate limiting step in the catabolism of haem. Its activity has been found to be present in almost all animal species and tissues (Maines, 1988). A schematic diagram of the pathway can be seen in Figure 1.1.

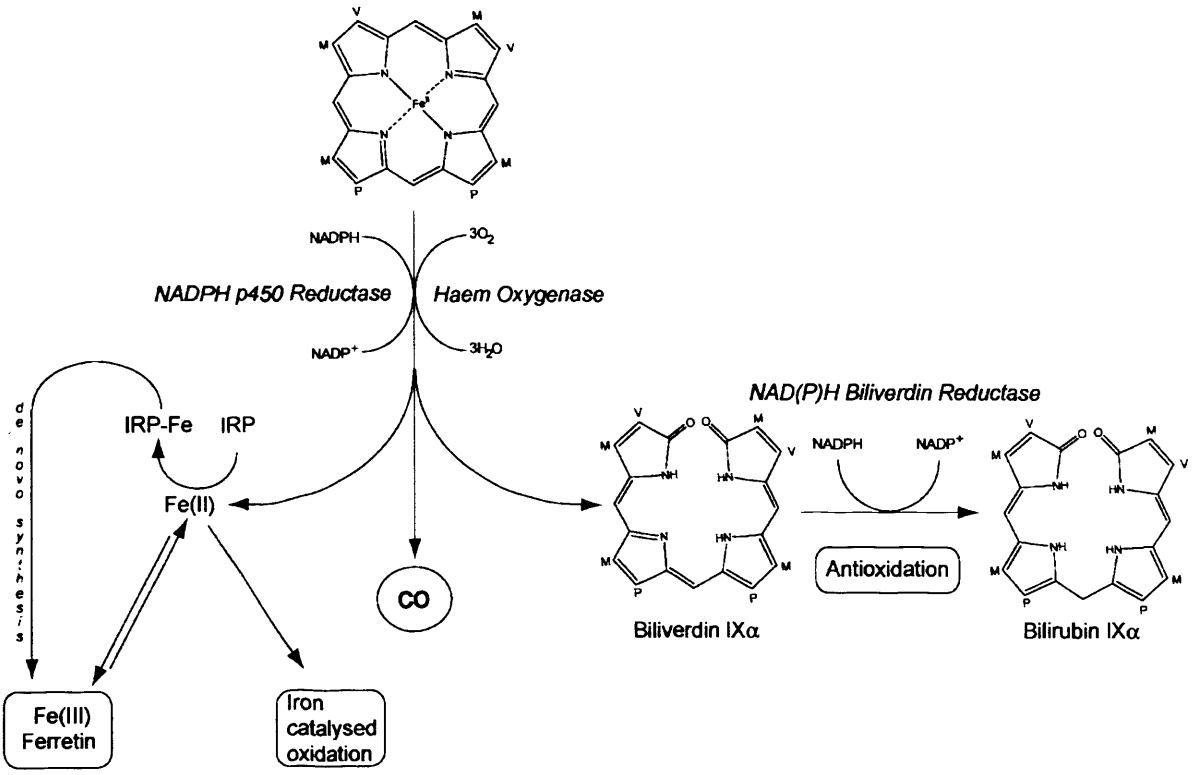


Figure 1.1:- Schematic representation of the haem oxygenase pathway. The diagram shows the major components of the pathway and the various enzymes involved in haem metabolism. Extract from Ryter *et al*, Mol Cell Biochem. 2002 May-Jun;234-235(1-2):249-63.

HO was only identified about 2 decades ago as a distinct microsomal entity that is regulated by metal ions (Maines 1997). It is anchored to the endoplasmic reticulum by a hydrophobic sequence of amino acids at the carboxyl terminus of the protein (Xia et al. 2002). Haem is oxidatively cleaved by HO to biliverdin, carbon monoxide (CO) is then generated and the ferrous iron (Fe) is released.

There are at present three identified forms of HO: HO-1 or the inducible enzyme (Maines et al. 1986); HO-2 or the constitutive isoform (Cruse and Maines, 1988); and HO-3 which unlike HO-1 or HO-2 is a poor haem catalyst and has yet to be investigated further (McCoubrey et al. 1997) (see Table 1.1)

Table 1.1:- Comparison of all three isoforms of haem oxygenase

Properties	HO-1	HO-2	HO-3
Cellular Localisation	Microsomes	Mitochondria	?
Chromosomal Localisation	22 q 12	16p13.3	?
Molecular Weight	32 KD	36 KD	33 KD
Tissue Distribution	Liver, kidney, heart, vascular smooth muscle, endothelium, lung and brain	Nervous system, vessels, testes, and intestine	Thymus, spleen, heart, testes, brain
Roles	Cellular homeostasis, anti inflammatory	Neural signalling and vascular regulation	?
Regulation	Haem, oxidative stress, heavy metals, nitric oxide, hypoxia	Glucocorticoids, opiates	?
Modified from (Maines 1997)			

HO-1, also known as heat shock protein (HSP) 32, is exquisitely sensitive, not only to metals but to many stimuli and agents that cause oxidative stress and by several pathological conditions e.g. heat shock (Ewing, Weber, & Maines 1993; Redaelli et al. 2001), ischaemia (Nimura et al. 1996; Maulik, Sharma, & Das 1996; Masini et al. 2003), glutathione (GSH) depletion (Fulton & Jeffery 1994; Hoekstra et al. 2003), radiation (Keyse & Tyrrell 1989; Reeve & Tyrrell 1999; Suzuki et al. 2002), hypoxia (Foresti et al. 2001; Appleton et al. 2002), hyperoxia (Motterlini et al. 1995a; Dennery et al. 1997; Taylor, Carraway, & Piantadosi 1998) and cellular transformations and disease states (Mayer 2000; Kyokane et al. 2001; Choi & Otterbein 2002). In fact, to date there is no other enzyme studied that is induced by so many diverse stimuli as HO-1. HO-2 on

the other hand is only known to be induced by adrenal glucocorticoids (Gcs); hence, its label as a constitutive isoform (Maines, Eke, & Zhao 1996; Raju, McCoubrey, & Maines 1997).

The catalytic activity of the HO system plays an important role in the maintenance of cellular haem homeostasis and haemoprotein levels, among which are the enzymes nitric oxide synthase (NOS) and soluble guanylate cyclase (sGC). HO also acts to protect the cell from the haem molecule, which promotes lipid peroxidation and oxygen free radical formation (Koehler & Traystman 2002). In addition to the action of HO itself, the products of its activity are also capable of performing various important functions. Indeed, the study on biliverdin, bilirubin, CO and Fe has intensified and many more biological roles are now attributed to these molecules. Both biliverdin and bilirubin have been shown to be potent antioxidants (Nakagami et al. 1993; Dore et al. 1999; Baranano et al. 2002b), while biliverdin has also been shown to inhibit viral replication (Mori et al. 1991). Similarly, Fe has been shown to be involved in the regulation of several genes including NOS (Anning et al. 1999; Otterbein et al. 2003c). CO, as will be discussed later, has developed into an important area of research undoubtedly becoming a keystone molecule in the physiology of life. This chain of events involving the products of HO is, however, triggered as a secondary line of defence. In fact, during normal metabolism, antioxidants such as superoxide dismutase (SOD), catalase and glutathione peroxidase are constitutively expressed and provide the baseline protection against oxidative stress. When this primary defence is overcome, the HO-1 pathway is activated and becomes a potent detoxifying system (Clark et al. 2000a; Foresti and Motterlini, 1999). Failure of HO-1 to be induced results in

inadequate cellular defence leading to cellular and tissue injury (Yachie et al. 1999).

Haem degradation is not specifically linked to HO. Haem can be degraded both chemically (coupled-oxidation) and enzymatically. Both mechanisms require oxygen (O_2) and a reducing agent needed for the activation of O_2 and the reduction of the haem iron from ferric (Fe^{3+}) to ferrous (Fe^{2+}) and/or maintenance of iron in the Fe^{2+} state (Atamna & Ginsburg 1995). The HO system uses nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent. NADPH activates O_2 by the donation of an electron thus initiating the multistep cleavage of the α, β, γ, and δ methene carbon bridges of haem. In the mammalian HO system only the α-methene carbon bridge is cleaved to form CO and biliverdin. Other minor pathways of haem breakdown include; xanthine oxidase and hydrogen peroxide (H_2O_2) plus NADPH-cytochrome P450 reductase (Lincoln, Aw, & Bonkovsky 1989; Balla et al. 1991). However, in these instances a mixture of pyrollic compounds and not biliverdin or CO are formed (Maines 1997; Koehler & Traystman 2002).

Vital for the functioning of HO is the microsomal enzyme NADPH-cytochrome P450 reductase. It transfers an electron from NADPH to haem and utilises oxygen for the cleavage of haem (Wang & de Montellano 2003). Haem is, as mentioned previously, specifically cleaved at the α-methene bridge. For each molecule of haem oxidized three molecules of O_2 and three molecules of NADPH are used. For each mole of O_2 used in the reaction one mole of water (H_2O) is produced making HO a mixed-function oxidase (Maines 1997).

It is interesting to note that HO-1 and HO-2 are products of two different genes, share little amino acid or nucleotide sequence and have little in common in

terms of amino acid composition, transcript number or size (Wilks 2002; Weng et al. 2003). HO-1 is the smaller of the two with a molecular weight of ~32 kDa, whilst HO-2 is about 36 kDa in size. As well as differing in gene organisation, structure and chromosomal localization both HO-1 and HO-2 vary in cell type, tissue distribution and regulation (Parfenova et al. 2001; Weng, Yang, Weis, & Dennery 2003). But it is the similarity in their mechanism of haem catalysis, substrate specificity and cofactor/coenzyme requirements that groups them. HO-1 and HO-2 are both single copy genes; however, HO-1 protein transcription requires only a single ~1.8 kb message, whereas HO-2 requires 2 or more transcripts (Zou et al. 2000; Scapagnini et al. 2002). These various HO-2 transcripts have been shown to arise from the usage of three different 5' untranslated regions (UTRs) and two different poly A signals with as many as five different transcripts being identified in the rat testes.

Interestingly, both HO-1 and HO-2 have been evolutionarily conserved in both primary amino acid and nucleotide sequences. This is best displayed by an >80% homology for HO-1 in rat, mouse and human whilst HO-2 cDNA showed over 90% conservation between rat, rabbit and human. There exists in both HO-1 and HO-2 a 24 amino acid long segment that is perfectly conserved in rat, rabbit and human with the only exception being a conservative substation of a leucine residue found in all HO-1 proteins for a methionine residue found in all HO-2 proteins. The flanking regions also display substantial amino acid sequence and structure similarity in both isoforms. The 24 residue segment itself is hydrophobic and forms a haem pocket that binds haem pyrrole ring 1 and 2 containing a vinyl side chain through electrostatic interactions, allowing isomer specific attack of the activated O_2 on the α-methene carbon bridge. The

consensus sequence Leu-Leu-Val-Ala-His-Ala-Tyr-Thr-Arg is highly conserved throughout nearly all species and is recognised as the signature of HO (Maines 1997; Wilks 2002). In HO-1, the histidine residue 132 is absolutely necessary for haem catalysis (Chu et al. 1999). The HO haem pocket only has specificity towards the side chains of the porphyrin ring, thus it does not recognize the metal moiety of the metalloporphyrins and hence non physiological metalloporphyrins such as zinc protoporphyrin (Zn-PP), tin protoporphyrin (Sn-PP) and others can compete with iron protoporphyrin (Fe-PP) and inhibit enzyme activity (Appleton et al. 1999). A facet which has been exploited to the fullest in studies determining the functioning and role of the HO pathway (Labbe, Vreman, & Stevenson 1999; Mancuso et al. 1999).

Differences in the regulation of both genes stem from the promoter regions. The promoter region of HO-1 has many regulatory elements containing the consensus sequences necessary for binding several regulatory factors such as; heat shock protein (hsp), activating protein-1 (AP-1), nuclear factor-κB (NF-κB), Nrf2 and metal regulatory elements (Alam, Camhi, & Choi 1995). Whereas, only a single glucocorticoid response element (GRE) is present and functioning in the HO-2 gene. GRE is not a strong transcriptional promoter and Gcs exerts a prominent regulatory effect on HO-2 expression at the protein level (Raju, McCoubrey, & Maines 1997).

HO-1 gene structure and regulation differ from that of other stress proteins. Unlike other HSP's HO-1 has four introns which are presumed not to be required for rapid transcriptional activation (Immenschuh et al. 1998). HO-1 induction by heat stress is a common and coordinated response in organs of

different species, reaching its peak after about 1 h in the heart, liver, kidney and brain again differentiating it from other HSP's.

An increase in HO-1 messenger ribonucleic acid (mRNA) transcription is accompanied by an increase in the binding of heat-shock nuclear factor(s) (HSF) to the promoter region of the gene and involves message stabilisation as a major factor in the accumulation of the ~1.8 kb transcript. Metals on the other hand, increase HO-1 primarily by increasing the transcription rate (Maines & Kappas 1976).

HO activity varies greatly among tissues with the highest HO activity being found in the spleen, testes and brain (Oshiro et al. 1999; Xia, Cui, Zhang, Shen, Wang, Li, Chen, & Yu 2002). Interestingly, the spleen is the only organ in which HO-1 is the predominantly expressed isozyme under normal conditions (Maines 1997). HO-2 is expressed in neuronal populations in the forebrain, hippocampus, midbrain, basal ganglia, thalamic regions, cerebellum and brain stem (Yamanaka et al. 1996; Scapagnini, D'Agata, Calabrese, Pascale, Colombrita, Alkon, & Cavallaro 2002). Throughout the brain many neurons that express HO-2 also express high sGC and cyclic guanosine 5'-monophosphate (cGMP); for instance, the purkinje neurons of the cerebellum, which normally don't express NOS, display high levels of cGMP (Baranano & Snyder 2001). Such high levels of HO expression and the lack of NO in certain neurons suggest that CO is a major signaling molecule in the hippocampus (Baranano & Snyder 2001). The hippocampal complex is a region of the brain involved in learning and memory and is an integral pathway for carrying impulses from the neocortex through dentate gyrus to other brain regions. Interestingly, pathophysiological changes in this region have been linked to various

neurological diseases and age related dysfunction (Scapagnini, D'Agata, Calabrese, Pascale, Colombrita, Alkon, & Cavallaro 2002).

Adrenal steroid HO-2 expression in the development stage of growth has been shown to be intimately linked with neurons involved in motor function and cognition, thus demonstrating a pivotal role of adrenal steroids in brain growth and differentiation (Maines 1997).

HO-1 and HO-2 have been shown to play an important role in the reproductive system as well (Mancuso et al. 1997a). Both isoforms show a cell type-specific pattern of expression as well as a developmentally linked expression of five different HO-2 transcripts. HO-2 has been shown to be expressed in a stage specific manner linking it to spermatogenesis, possibly providing iron which is essential for the process to take place. It has also been implicated to play a part in signal transduction and cell cycle events (Mancuso et al. 1997b; McLean et al. 2000). The presence of both isozymes throughout the reproductive system is evidence in itself of the role of HO isozymes in the control of the reproductive system function.

In the cardiovascular system, HO-2 is predominantly expressed under normal conditions. It is worth noting that under conditions of stress the increase in HO-1 mRNA expression in the heart, kidneys and vasculature is significant (Otterbein & Choi 2000; Morse & Choi 2002). HO-2 has been detected in blood vessel wall constituents (endothelial and smooth muscle cells), connective tissue elements (fibrocytes and fibroblasts), visceral smooth muscle cells, mesothelial cells of serous membranes, select epithelial cell populations, carotid body chemoreceptors and in the adventitial neurons of blood vessels (Siow, Sato, & Mann 1999). The arterioventricular (AV) node of the heart and myocytes

expresses particularly high levels of HO-1 when stressed. CO generated by HO activity in the heart has been suggested to regulate cGMP production (Duckers et al. 2001; Liu, Song, & Lee 2001). Interestingly, oxidative stress of the kidney (ischemia) is translated into a prominent and sustained increase in HO-1 mRNA and protein expression in the heart and aorta, suggesting an intimate link between these organs (Maines & Kappas 1976). It is also important to note the absence of HO-2 from striated muscle and that it appears to be coexpressed with NOS in vascular endothelial cells and in certain parasympathetic and sensory ganglia nerve cells (Maines 1997).

As with the brain and testes, the liver displays developmentally regulated HO expression (Maines 1990). However, the total HO activity decreases as the newborn matures. This is due to an initial surge in HO-1, which rapidly declines during maturity and is superseded by a gradual increase in HO-2 levels. Regulation of both HO-1 and HO-2 were first compared in the liver and it is known that HO activity due to the induction of HO-1 can be increased up to 100 fold in the presence of oxidative stress inducing compounds without a concomitant increase in HO-2 (Maines 1997). CO, meanwhile has been shown to function as an endogenous modulator of vascular perfusion in the liver suggesting that the huge increase in HO-1 when exposed to oxidative stress inducing compounds may be a pathophysiological factor in liver disease (Suematsu, Wakabayashi, & Ishimura 1996b).

The sources of haem for HO mediated degradation are plentiful: cytochrome and tissue haemoproteins; hemoglobin; myoglobin or newly synthesized free haem (prior to apoprotein incorporation). But why does the haem molecule become available for haem degradation? We must consider a number of factor

to understand the answer: i) all tissues synthesise haem and part of newly synthesized haem is readily degraded by HO (Maines & Kappas 1976; Maines 1980); ii) under conditions of stress, non-HSP synthesis is all but halted leading to a decreased demand for haem and thus more availability of free haem for degradation; iii) cellular membranes being susceptible to damage by free radicals and changes in redox potential in the vasculature allow access to circulating hemoglobin (Maines, Chung, & Kutty 1982; Maines et al. 1993); and iv) haemoproteins are often denatured by free radicals (Maines & Kappas 1975; Trakshel, Sluss, & Maines 1992). Examples of such denatured proteins include cytochrome P420, myoglobin and cytochrome c haem. With so many sources of haem for HO activity under normal unstressed conditions, HO-2 is likely to utilize free haem and haem of cellular haemoproteins. While during conditions of stress HO-1 may find haem from additional sources that affect the rate of haemoprotein denaturation e.g. cytochrome P450 to P420 conversion, hemoglobin, myoglobin denaturation, causing an availability of extracellular haem for degradation.

As such, HO-1 may serve as a primary line of defence in the cellular scenario. Its activation and subsequent degradation of the prooxidant haem molecule result in the generation of the antioxidant bile pigments and CO. In addition the iron released would induce ferritin synthesis, the iron storage protein; iron being an effective catalyst for lipid peroxidation (Eisenstein et al. 1991). The haemoprotein NOS would be subject to all the factors effecting other haemoproteins stability, synthesis and turnover. Thus, stress-induced CO production could overcome the attenuated ability of the cell to generate cGMP as NO production is decreased thus playing a role in cellular defence, therefore,

HO-2, could function in the maintenance of basal cGMP production alone or in conjunction with NOS depending on the cell type and tissue.

The similarity between the HO system and NOS pathway is undeniable. Both HO and NOS enzymes have been clarified in a constitutive and an inducible form, the inducible form of each responding to very similar inducers e.g. bacterial endotoxins, cytokines and reactive oxygen intermediates. Both inducible forms are also present and active in many organs and tissues and their induction involves gene activation and de novo protein synthesis. Interestingly, Gcs has an opposing effect on expression of the two proteins in the brain with HO increased and NOS decreased. Both HO-1 and iNOS are however highly upregulated in cells involved in the resolution of inflammation e.g. macrophages, neutrophils, polymorphonuclear and mononuclear cells. The similarities between NO and CO suggest a possible sharing in the control of the relaxation processes (Foresti and Motterlini, 1999).

An X-ray crystal structure of human HO-1 in complex with its substrate haem can be seen in Figure 1.2. Interestingly, a direct link between HO-1 induction and NO has been demonstrated. Indeed, NO has been shown to up-regulate the expression of HO-1 in a variety of tissues and cells (Foresti and Motterlini, 1999; Motterlini et al. 2000; Sammut et al. 1998; Foresti et al. 1999; Vesely et al. 1998; Clark et al. 1997; Foresti et al. 1997; Motterlini et al. 1996; Motterlini et al. 1996) (Sawle et al. 2001). The range of NO releasing agents capable of stimulating HO-1 induction is vast, including chemicals such as sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP). Furthermore, the level of HO-1 induction appears to be directly related to the decomposition rate of the NO donor.

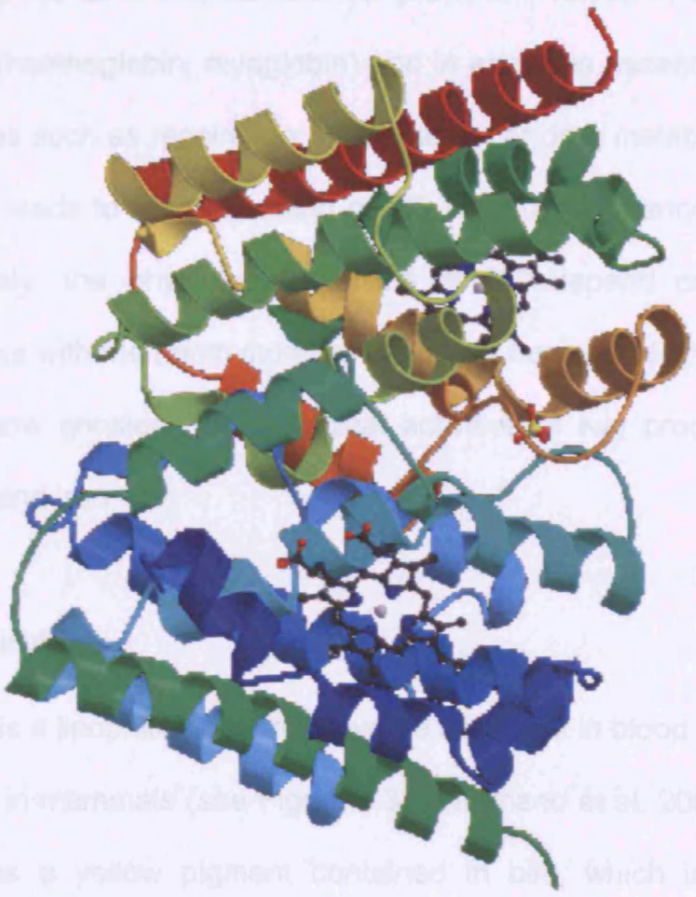


Figure 1.2:- X-ray crystal structure of human HO-1 in complex with its substrate haem captured via X-ray diffraction.
The figure was rendered using Protein Data Bank 3DB Browser, which is available on line at http://pdb-browsers.ebi.ac.uk/pdb-bin/pdbmain.

As eluded to earlier, HO-1 itself is not a cytoprotective molecule but the products it generates from haem degradation are. The activation of the HO-1 pathway results in the production of the powerful antioxidants biliverdin and bilirubin (Clark et al. 2000a; Clark et al. 2000b; Foresti et al. 1999) and the vasoactive CO (Sammut et al. 1998; Motterlini et al. 1998).

The physiological need for this pathway is highlighted by the numerous inducers of HO-1 known to date and the sensitivity of the pathway to a diversity of stimuli. Such a system allows for a rapid up-regulation of cellular defences in response to a threatening situation, such as the presence of a strong oxidant.

Haem serves as a vital cofactor for proteins involved in oxygen transport and storage (haemoglobin, myoglobin) and in enzymes essential for critical cellular processes such as respiration, inflammation or drug metabolism. Haemoprotein turnover leads to the production of CO as a consequence of haem utilisation. Conversely, the physiological effects of CO depend on its ability to form complexes with the haem moieties of cellular haemoproteins.

Let us now consider the biological activities of two products of HO activity; bilirubin and iron.

1.2 Bilirubin

Bilirubin is a lipophilic linear tetrapyrrole abundant in blood plasma which occurs uniquely in mammals (see Figure 1.3) (Baranano et al. 2002a). Bilirubin is best known as a yellow pigment contained in bile, which is released from the gallbladder into the duodenum to aid digestion. Hipocratic medicine considered yellow bile as one of the four bodily fluids or humors that promoted health when in balance and illness when not. An excess of yellow bile was believed to make one choleric (easily irritated; irascible, inclined to anger), whilst, in China bilirubin has long been used in traditional Chinese medicines (Niu Huang) as a potent fat soluble antioxidant (McDonagh 2001). It was not until the middle of the last century that the chemical structure was determined and the synthesis of bilirubin achieved. Hans Fisher, a German chemist, was awarded the Noble Prize for his work on the bilirubin precursor haem. The difficulty to work with and obtain a pure yield as well as the inherent instability of both biliverdin and bilirubin compared to haem, meant it was not until recently that scientists began to study them in any real depth.

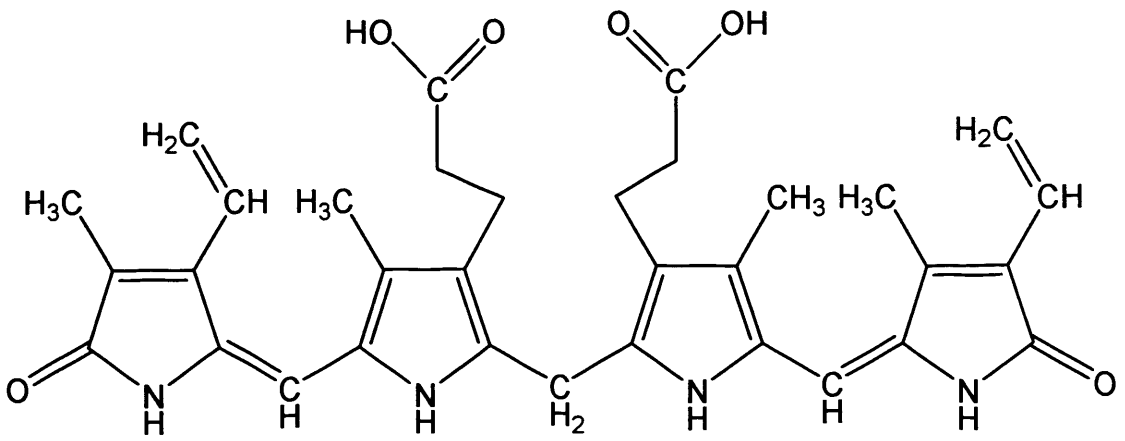


Figure 1.3:- The linear, tetrapyrrole chemical structure of bilirubin.

Bilirubin formation is catalysed by biliverdin reductase (BVR) (Salim, Brown-Kipphut, & Maines 2001). BVR is coexpressed in cells that display HO-1 and/or HO-2 under normal conditions as well as in regions and cell types that have the potential to express heat shock inducible HO-1. Bilirubin, a bile pigment and the reduced form of biliverdin, is an important product of haem degradation. Both bilirubin and biliverdin are produced in adults at the rate of ~0.25g per day (McDonagh 2001). The pathway is vital for the homeostasis of haem, for the biosynthesis of plant and algal pigments, and in animals it appears to play a key role in oxidative stress defence (Greenberg 2002; Baranano, Rao, Ferris, & Snyder 2002a).

The reason for the production of bilirubin in mammals is intriguing. Why convert biliverdin IXα, a non-toxic, easily excretable waste product into bilirubin, a substance that is less water soluble, neurotoxic, seeds gallstones and has to be further metabolised for disposal? Although there is not a definitive answer as yet, emerging evidence suggests that bilirubin is a potent regeneratable lipid-soluble antioxidant which protects against free radical and oxy-radical damage both systemically and at local sites of injury *in vivo* (Galbraith 1999; Ryter &

Tyrrell 2000; Clark et al. 2000c; McDonagh 2001). Bilirubin has been shown to possess several biological activities: antioxidant, antimutagen, anti-complement and cytoprotector (Clark et al. 2000a; Clark, Foresti, Sarathchandra, Kaur, Green, & Motterlini 2000c). In fact, bilirubin, is the most powerful endogenous antioxidant known *in vitro* and is also a potent physiological antioxidant *in vivo*.

The antioxidant properties of bilirubin have been associated with a reduced risk of coronary artery disease (CAD) in individuals with a mildly increased serum bilirubin level (Hopkins et al. 1996; Mayer 2000). The extent of this effect is comparable to that of HDL-cholesterol. Interestingly, individuals who smoke significantly lower their serum bilirubin level and attenuate the protective effect of the antioxidant (Van Hoydonck, Temme, & Schouten 2001).

It has been suggested by Dennery *et al* that neonatal hyperbilirubinemia could be a transitional antioxidative mechanism in the circulation of human neonates (Dennery et al. 1995). The authors showed thiol barbituric acid reactive substances (TBARS) in Gunn rats exposed to hyperoxia were significantly decreased in jaundice pups, while, other serum antioxidants (catalase, glutathione (GSH), vitamins A, C, E and uric acid) remained the same in jaundice and non-jaundice groups.

Bilirubin and the bilirubin-albumin complex present in serum are scavengers of O_2^- and peroxyl radical, their potency increasing with decreasing pH. The ability of bilirubin to scavenge peroxyl radicals is much higher than that of serum albumin and of the same order as the bilirubin-albumin complex found in serum. Studies have also demonstrated that free and albumin bound bilirubin are efficient co-antioxidants for α-tocopherol (Young & Caughey 1986; Day et al. 1999). For example, the antioxidant activity of bilirubin on oxidation of low-

density lipoprotein (LDL) is mediated by interaction of the bile pigment with LDL's α-tocopherol. It has also been demonstrated that bilirubin protects its carrier protein, serum albumin, from oxidative damage exerted by hydroxyl (OH•) radicals (Adhikari & Gopinathan 1996). Along with ascorbate and urate, bilirubin is a very important antioxidant in plasma rivalling vitamin E in lipophilic microenvironments e.g. lipoproteins, membranes. It has also been speculated that bilirubin may be an important cytoprotector for tissues which are less equipped for antioxidant defence like myocardium and nervous tissue (Otterbein & Choi 2000; Baranano & Snyder 2001).

Interestingly, bilirubin has been shown to possess antimutagenic properties by inhibiting the direct acting mutagenicity of 4-nitriquinoline-1-oxide (4NQO) in strain TA100 of salmonella typhimurium (Camoirano et al. 1994). Biliverdin also possesses this ability but to a lesser extent due to bilirubin's greater nucleophilicity. While conjugated bilirubin and biliverdin show an inhibitory effect on complement dependent reactions *in vitro* blocking the complement cascade, especially at the C1 step (Nakagami, Toyomura, Kinoshita, & Morisawa 1993; Arriaga, Mottino, & Almara 1999).

Were conversion of haem to biliverdin solely a route for disposal of metabolic waste, as once thought, it would hardly matter which particular isomer is produced since all are easily excreted. However, only one of the four, the isomer IXα, generates a lipophilic rubin on reduction and the same isomer is the only one that can serve as a biosynthetic precursor for the phytochromobilins and phycobilins of plants, algae, and cyanobacteria (McDonagh 2001). Thus, it is hardly surprising that HO cleaves the haem with such high regiospecificity for

the α-methene carbon bridge. Nevertheless, very small amounts of the other three isomers, particularly IXβ, are produced in humans and other mammals.

As mentioned, bilirubin is potentially neurotoxic. When hepatic bilirubin glucuronosyl transferase activity is low or absent, as in newborn babies or rare genetic disorders, the pigment accumulates in the circulation bound to albumin. If the albumin binding capacity is compromised, excess bilirubin may partition into the brain and cause irreversible damage or even death. Each year countless thousands of babies are treated for hyperbilirubinemia and an unfortunate few die or are left permanently injured (Baranano & Snyder 2001; Greenberg 2002). Conversley a neuroprotective role for bilirubin has also been demonstrated. Dore *et al* showed that H_2O_2 toxicity was increased in hippocampal neuron cultures from HO-2 knockout mice (Dore, Takahashi, Ferris, Hester, Guastella, & Snyder 1999). Upon addition of free bilirubin or albumin-bilirubin conjugate there was an improvement in survival. They also showed that after focal cerebral ischemia, induced by occlusion of the middle cerebral artery followed by repurfusion or intracerebral injection of the excitotoxic amino acid N-methyl-D-aspartate, injury was more extensive in HO-2 knockout mice than in wild type mice, which is consistent with a role of HO-2 products in protection from ischemia (Dore, Takahashi, Ferris, Hester, Guastella, & Snyder 1999).

Bilirubin is responsible for the yellow discolouration of the skin in jaundice in newborns (Stevenson et al. 2001; Baranano, Rao, Ferris, & Snyder 2002a). Erythroblastosis fetalis, a condtion which usually occurs from Rhesus (Rh) blood group incompatibility between mother and neonate, results in the accumulation of bilirubin due to antibody induced hemolysis with the

subsequent degradation of free haem to bilirubin (Mukhopadhyay et al. 2003). Treatment involves phototherapy and an exchange transfusion to prevent kernicterus in which high concentrations of bilirubin gain access to the basal ganglia and other brain structures resulting in lethargy, rigidity, seizures, death and in long term survivors, choreothetosis, hearing loss and Parinaud's syndrome (paralysis of upward gaze). Hepatocellular disorders and extrahepatic biliary obstruction can also result in jaundice where excess bilirubin in the blood becomes deposited in elastin rich tissues such as skin and sclera (Greenberg 2002).

Classically bilirubin is seen at sites of recent bleeding, such as haematomas under the skin – commonly known as bruising (seeFigure 1.4) (Beschorner et al. 2000). Yellow colouration of cerebrospinal fluid is also a sign of subarachnoid haemorrhage.

Bilirubin has been shown to be an antioxidant capable of scavenging peroxyl radicals and has been reported to protect against a variety of pathological processes including complement-mediated anaphylaxis (Nakagami, Toyomura, Kinoshita, & Morisawa 1993), myocardial ischemia (Marilena 1997), pulmonary fibrosis (Choi & Alam 1996) and cyclosporine nephrotoxicity (Polte et al. 2002).

Kernicterus pathophysiology exemplifies the toxicity of bilirubin. This toxicity is achieved at micromolar concentrations, causing death of cultured neurons and cerebral microvascular endothelial cells *in vitro*, which has features of apoptotic cell death. Specific glutamate receptors have been implicated in the pathogenesis of bilirubin toxicity as N-methyl-D-aspartate (NMDA) receptor antagonists can protect neurons from its toxicity (McDonald et al. 1998).

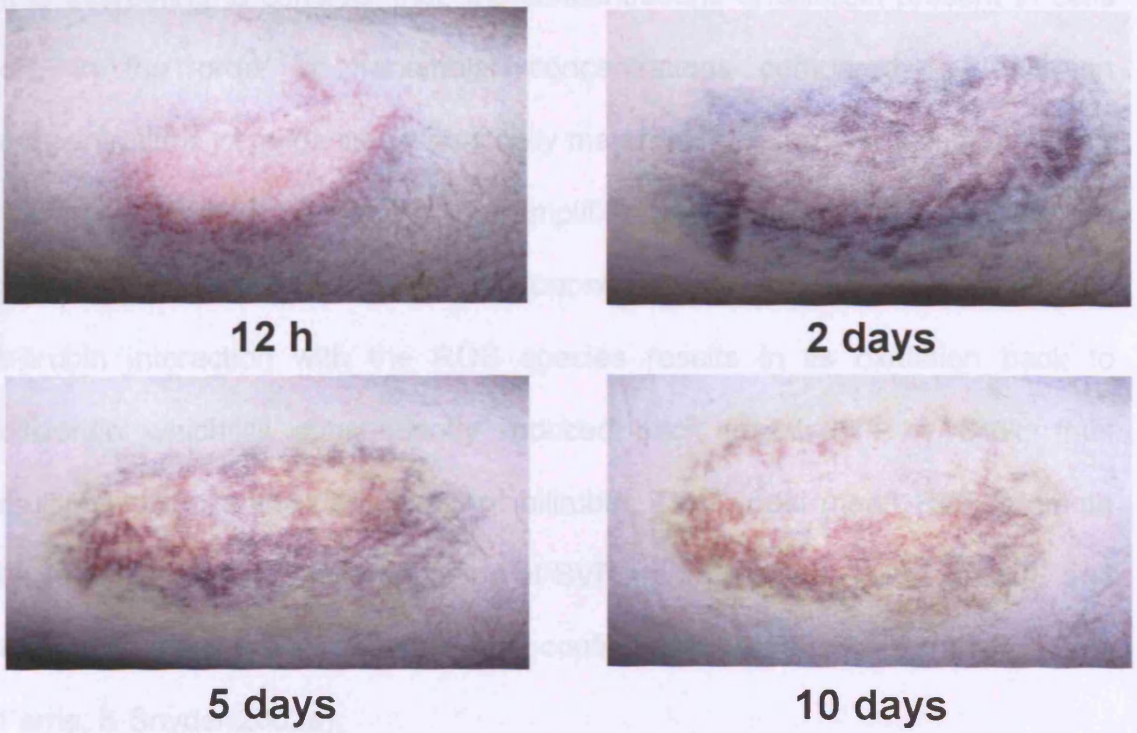


Figure 1.4:- Resolution of a sub-dermal haematoma.
Initial colouration is due to the presence of blood in the wound. As the haem is degraded we can see the greenish hue of biliverdin and finally the yellow pigmentation associated with bilirubin.

Takahashi *et al* identified proteins that interact with HO-2 (Takahashi et al. 2000b). They found one such protein, amyloid precursor protein (APP), which is the source of β-amyloid in Alzheimer's disease and is mutated in some familial forms of the disorder. Interaction of wild type APP with HO-2 inhibited the activity of HO-2 suggesting an intriguing connection between HO-2 and Alzheimer's disease. Moreover, cortical neuron cultures expressing the Swedish mutation showed bilirubin production defects and enhanced toxicity from H_2O_2, suggesting that HO may help to regulate the oxidative injury in Alzheimer's which is one way that haem deficiency could impact the disease (Takahashi et al. 2000a).

It is interesting to consider that, the concentrations of bilirubin present in cells are in the order of nanomolar concentrations compared to the high concentrations of oxidants which locally may reach micromolar levels. It would, therefore, seem a mechanism that amplifies the antioxidant effect of the bile pigment is required. Barańano has proposed a redox cycling process whereby bilirubin interaction with the ROS species results in its oxidation back to biliverdin which is subsequently reduced back to bilirubin by BVR, thus multiplying the antioxidant effect of bilirubin. This would mean ROS promote biliverdin synthesis, while, depletion of BVR would increase levels of ROS and their toxic effects, both of which were confirmed by Barańano (Baranano, Rao, Ferris, & Snyder 2002a).

Barańano *et al* have also shown that as little as 10 nM bilirubin protects against almost 10,000 fold higher concentrations of H_2O_2 indicating that bilirubin cannot be acting simply as a stoichiometric antioxidant against H_2O_2. As such, bilirubin acts in a catalytic fashion whereby bilirubin oxidised to biliverdin is rapidly reduced back to bilirubin a process that could readily afford 10,000 fold amplification. Thus, a redox cycle based on BVR activity provides physiological cytoprotection as BVR depletion exacerbates the formation of ROS and augments cell death (Baranano, Rao, Ferris, & Snyder 2002a).

Cells depleted of GSH or BVR indicate that BVR is of comparable or greater importance than GSH in physiological cytoprotection. Several parallels can be drawn between the bilirubin redox cycle and the cycling of GSH, another chain breaking antioxidant. Both involve a single chemical that exists as either an oxidised or reduced form, in both cases the reduced form predominates under healthy conditions. Both are also part of a concerted stress response, including

heat shock proteins, that are induced when cells are challenged with a stressful insult. Disruption to either the BR/BV or GSH cycle results in an increase in oxidative stress and consequently cell death. However, while bilirubin levels are well below 100 nM, GSH concentrations are in the millimolar range. Interestingly though, the abundance of BVR activity in all tissues indicates bilirubin cycles at a much higher rate than GSH. There are also, however, some major differences between the two cycles. For instance, biliverdin is oxidised directly to bilirubin unlike GSH which requires the presence of a peroxidase, a reductase and distinct enzymes for synthesising it. Conceivably, bilirubin and GSH provide physiological antioxidant activities for distinct types of intracellular molecules. Bilirubin, which is highly lipophilic, is intimately associated with cell membranes where it might prevent lipid peroxidation and protect membrane proteins from oxidative damage. On the other hand, GSH is highly water-soluble and might be expected to protect cytoplasmic constituents (Baranano, Rao, Ferris, & Snyder 2002a).

Biliverdin reductases are unusual, possibly unique, in their ability to use either NADPH or NADH as cofactors, with a different pH optimum for each cofactor. They catalyse regiospecific reduction of just one of some fifteen double bonds in the biliverdin substrate and they are selective for particular biliverdin isomers. The location of the propionate side chains of biliverdin being key to turning it into bilirubin (Baranano, Rao, Ferris, & Snyder 2002a).

BVR may also have a special use in the placenta of mammals. In adults, bilirubin IXα has to be converted to more polar glucuronide conjugates by bilirubin glucuronosyl transferase or to photoisomers for excretion by the hepatobiliary system. In the foetus, the activity of glucuronosyl transferase is

low. Since neither biliverdin nor bilirubin glucuronides cross the placenta as readily as the much more lipophilic bilirubin, reduction of biliverdin to bilirubin facilitates the disposal of haem derived pigment across the placenta and prevents their accumulation in the foetus. High proportions of bilirubin IXβ (up to 87% of total bilirubin) occur in foetal bile (Blumenthal et al. 1980). This has been taken as evidence that haem catabolism *in utero* is different and less regiospecific than adults. However, it has been suggested that the IXβ isomer in foetal bile represents a tiny fraction of the total haem catabolised and that its relatively high concentration is simply a consequence of the different placental permeabilities of the IXβ and IXα isomers.

Continuing with the products of haem catabolism we now consider iron, while CO will be discussed later in the Introduction.

1.3 Iron

Iron is vital for all living organisms; however, excess iron can be lethal as it facilitates free radical formation. A newborn infant has a total body iron of about 250 mg. During growth iron absorption must exceed iron loss by ~0.5 mg per day in order to maintain a body iron concentration of about 60 parts per million. An adult male (70 kg) has a total body iron of about 4 g which remains constant throughout adult life (Conrad & Umbreit 2002). Thus, iron absorption is carefully regulated to maintain an equilibrium between absorption and body loss of iron. Much of the body iron is derived from dietary haem since haem binds few of the dietary chelators that bind inorganic iron in the gut. This is especially true in countries where meat is a significant part of the diet. The iron present in haem is released by HO to enter plasma as inorganic iron. Ferric iron is then

absorbed via a β_3 integrin and mobilferrin pathway (IMP). The association of IMP with divalent metal transport-1 (DMT-1) in the cytoplasm produces a large protein complex called paraferritin which serves as a ferrireductase. Paraferritin solubilises iron binding proteins and reduces iron to make iron available for production of iron containing proteins such as haem (Conrad & Umbreit 2002).

Too little absorption or too great a loss of iron results in iron depletion while too much absorption or too little loss results in hemosiderosis and hemochromatosis.

It is interesting to note that human subjects conserve iron more efficiently than other animals. Typical examples of iron loss from the body include: haemorrhage, desquamated epithelium of skin, intestinal cells, and intestinal secretions. Tissue iron stores generally dictate the level of iron absorption, e.g. the rate of erythropoiesis and hypoxia, while haemorrhage, haemolysis or hypoxia accelerated RBC production and rapid plasma iron turn-over also increase iron absorption. Diminished erythropoiesis, blood transfusion, return to sea level from high altitude and starvation all decrease iron absorption.

It has been demonstrated by numerous laboratories that vertebrates possess an anti-infective/anti-neoplastic defence system which has been termed "iron withholding". Very simply, the system attempts to deny availability of iron to invading microbial and neoplastic cells while permitting normal host cells access to the metal (Weinberg 1992). Both lactoferrin and transferrin, two powerful iron binding proteins, are constitutive components of the system found in all bodily fluids except urine. At times of microbial or neoplastic cell invasion these components are activated by the release of inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α). The defensive

response includes: i) suppression of intestinal assimilation of iron, ii) retention of iron by macrophages that have digested haemoglobin from decaying erythrocytes, iii) synthesis of ferritin to permit enhanced intracellular storage of the metal, iv) mobilisation of neutrophils to sites of invasion of extracellular bacteria with release of the contents of cytoplasmic granules that contain apolactoferrin, v) scavenging of iron at invasion sites by apolactoferrin and acquisition of the ferrated proteins by macrophages, and vi) synthesis of immunoglobulins against protein components of microbial uptake systems (Weinberg 1992). This system is highly effective against extracellular microbial pathogens but less useful in combating bacteria, fungi and protozoa that act intracellularly. However, additional methods of iron modulation and metabolism exist to counteract this further threat. This set of mechanisms also known as "iron depletion" includes: i) the removal of non-haem iron from the invading cells and from infected host cells via the use of reactive nitrogen intermediates derived from nitric oxide synthase, which destroy iron-sulphur centres, and ii) the reduction in net uptake of iron into infected host cells via down-regulation of expression of transferrin receptors (TfRs) (Weinberg 1992).

HO-1 releases ferrous iron (Fe^{2+})during the catabolism of haem. The release of Fe^{2+} from the core of the haem molecule leads to the rapid expression of the iron-sequestering protein ferritin as well as activating an ATPase pump which actively removes intracellular iron from the cell. The extracellular iron chelator desferoxamine mesylate (DFO) or the intracellular iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) perform some of the iron-depleting functions associated with ferritin (Otterbein et al. 2003b). Ferritin sometimes appears to subsume the role of HO-1 and other times it does not. Ferritin expression was

originally reported to be the mechanism by which HO-1 conferred resistance to oxidative stress in endothelial cells (Friebe et al. 1998). In addition, ferritin mediated the protection seen with HO-1 induction by haem in a model of hyperoxic lung injury (Taylor, Carraway, & Piantadosi 1998). In contrast, the potent cytoprotection provided by HO-1 induction in a model of endotoxic shock in rats appeared to be ferritin-independent as apoferritin and iron dosing were ineffective at conferring protection (Otterbein et al. 1997). The ATPase pump that actively removes intracellular iron from the cell contributes to decreasing the overall intracellular pool of Fe^{2+} and protecting the cells from apoptosis. The contribution of both the iron pump versus ferritin to the overall cytoprotective effect of HO-1 is unclear, but both are crucial mechanisms for preventing oxidative damage in a variety of situations in which HO-1 expression is increased.

Iron is also present in the protoporphyrin ring of sGC, a molecule that facilitates many of the actions of HO.

1.4 Soluble Guanylate Cyclase

The soluble form of guanylate cyclase, sGC is a haemoprotein (Maines 1997; Koesling 1999). The enzyme is a dimer of two similar but not identical peptides that bind two haems per dimer (see Figure 1.5). It is known that the presence of the tetrapyrrole ring is required for the catalytic activity of sGC and there is evidence to suggest that iron protoporphyrin (Fe-PP) is involved in the enzymes activity and binding of NO (Ignarro, Barrot, & Wood 1984).

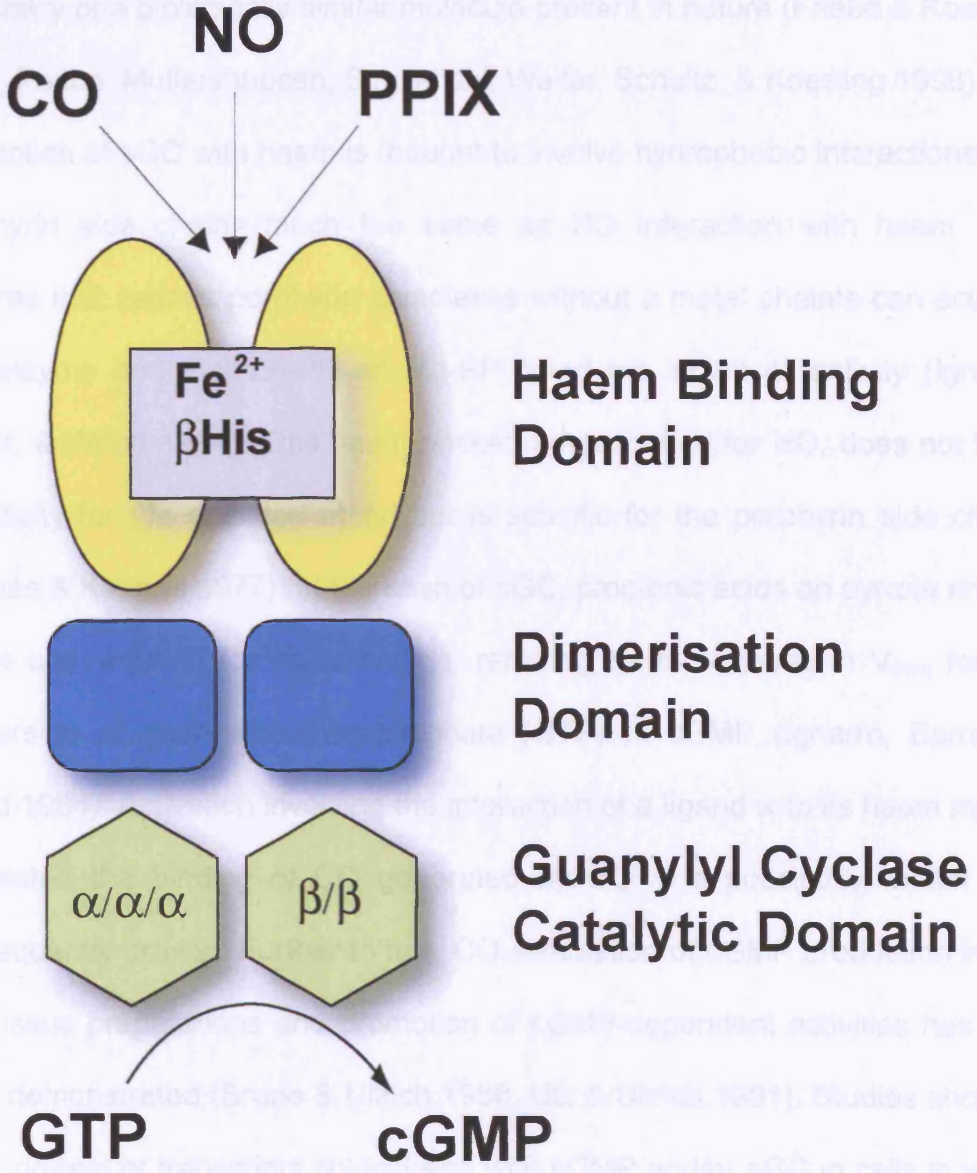


Figure 1.5:- Schematic structure of soluble guanylate cyclase showing known stimulators of the enzyme.
PPIX = protoporphyrin IX. Extract from Alexis Biochemicals Guanylyl cyclase/gaunosine phosphate metabolism web page.

CO can also bind to the haem iron activating soluble guanylate cyclase, although with an affinity much lower compared to NO (Friebe et al. 1996). However, the presence of YC-1, a benzylindazole derivative, has been shown to increase the affinity of sGC for CO, *in vitro*, by 4000% and there exists the

possibility of a biologically similar molecule present in nature (Friebe & Koesling 1998; Friebe, Mullershausen, Smolenski, Walter, Schultz, & Koesling 1998). Interaction of sGC with haem is thought to involve hydrophobic interactions with porphyrin side chains much the same as HO interaction with haem. This ensures that various porphyrin complexes without a metal chelate can activate the enzyme and that Zn-PP an Mg-PP could not inhibit its activity (Ignarro, Barrot, & Wood 1984). This haem pocket, as described for HO, does not have specificity for the chelated metal but is specific for the porphyrin side chains (Maines & Kappas 1977). In the case of sGC, propionic acids on pyrrole rings 3 and 4 are required for its activation, referring to its increase in V_{max} for the conversion of guanosine-5'-triphosphate (GTP) to cGMP (Ignarro, Barrot, & Wood 1984). Activation involving the interaction of a ligand with its haem moiety suggested the binding of CO generated by HO is a possibility, which was subsequently proven. Further to this, CO stimulation of cGMP production in cell and tissue preparations and promotion of cGMP-dependent activities has also been demonstrated (Brune & Ullrich 1988; Utz & Ullrich 1991). Studies showing HO-2 protein or transcripts colocalising with cGMP and/or sGC in cells in which NOS is nearly absent are consistent with sGC activation by HO generated CO (purkinje neurons) (Maines 1997; Koesling 1999). The effect of metalloporphyrins on both HO and sGC requires that they are used in sufficiently low concentrations to discriminate between HO and sGC inhibitions. Obviously HO has to be transcribed before it can act. One of the principal methods of transcription is via the mitogen activated protein kinases (MAPK).

1.5 Mitogen Activated Protein Kinases

1.5.1 Overview of the Cascade

The MAPKs are a large family of proline-directed serine/threonine kinases that require tyrosine and threonine phosphorylation of a ThrXTyr motif in the activation loop for activation (Torres 2003). Receptor ligand activation leads to activation of a phosphorylation cascade where the minimal module is formed by MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). MAPK and their activation cascades are critical pathways connecting extracellular ligands to the transcriptional machinery. MAPKs have been shown to be part of well conserved signalling pathways that control proliferation, differentiation, embryogenesis and cell death (Cobb & Goldsmith 1995; Torres 2003).

Four separate MAPK and activating cascades have been identified in mammalian cells and are activated by separate kinase cascades. These are: i) the classical extracellular signal regulated kinase (ERK, TEY) ERK1/2; ii) the c-jun N-terminal kinase (JNK, TPY) JNK1/2/3; iii) the p38MAPKα/β/γ/δ (TGY), the latter two families also being referred to as the stress activated protein kinases (SAPK) and iv) ERK5 (TEY) also called Big MAPK/BMK1 because of its high molecular mass (Cobb & Goldsmith 1995; Torres 2003).

The activation step of MAPKs are very specific and are carried out by dual specificity kinases, the MAPKK, which are themselves activated by phosphorylation by MAPKKK. Thus MAPK are the terminal kinases of a three tiered module of kinases that are sequentially activated by a variety of stimuli acting through diverse receptor families (Torres 2003).

ERK was the first mammalian MAPK to be identified. It is a 42 kDa protein which increases tyrosine phosphorylation upon stimulation with mitogens,

cytokines and ligands for G-protein linked receptors hence the name ERK to reflect its diversity of regulators. All of the components of the ERK module, ERK1 or ERK2, MEK1 or MEK2 and Raf isoforms, have been known for over a decade although questions still remain relating to the function and regulation of each component. The ERK pathway mediates cellular responses to growth and differentiation factors including platelet derived growth factor, epidermal growth factor and IL-5 (Cobb & Goldsmith 1995; Torres 2003).

JNK and p38MAPK are primarily activated by cellular stresses such as inflammatory cytokines although they have also been shown to be regulated by UV and γ-irradiation. Much still remains to be elucidated about JNK pathways but homologous recombination of JNK genes in mice has shown that, although viable, the mice are defective in apoptotic and immune responses. A double knockout mouse model of JNK1 and JNK2 resulted in embryonic death. Gene targeting of p38 resulted in various outcomes although all mice died during embryonic development. Two MAPKK have been identified as the kinases phosphorylating JNK (MKK4 and MKK7). Studies have suggested that the cooperation of both MKK4 and MKK7 is required to activate JNK in response to environmental stress. In contrast only MKK7 is required for TNK activation of JNK (Cobb & Goldsmith 1995; Torres 2003).

The p38MAPK are activated by the dual specificity kinases MKK3 and MKK6. Only MMK3 is required for TNF-α induced p38 activation and cytokine expression.

Both JNK and p38MAPKs are activated by stress related stimuli such as heat shock, inflammation, hyperosmolarity, UV and, γ-irradiation (Cobb & Goldsmith 1995; Torres 2003).

Activation of the MAPKs must be terminated at a precise time for proper cell function as the duration and extent of MAPK activation which is governed by the equilibrium between the activity of kinases and MAPK phosphatases (MKP) may determine the biological outcome.

The MAPKs play an important role in the regulation of gene expression either by directly phosphorylating transcription factors or by activating other kinases with transcription factors as substrates. The consequences of phosphorylation on the function of transcription factors are multiple; i) it can regulate activation, DNA binding ability, and association with co-regulators; ii) it can regulate protein stability by preventing ubiquitination thus prolonging half-life; and iii) it can control the subcellular localization (see Figure 1.6) (Torres 2003).

The essential role of ROS involvement in bacterial killing has been extensively documented in the literature. This ROS involvement seems to require tyrosine phosphorylation. Stimulation of macrophages with zymosan activated serum results in an increase in ROS and subsequent ERK1/2 activation, which requires the presence of H_2O_2. Other studies have shown that ROS produced by phagocytosis of fibres in rat alveolar macrophages induced activation of ERK and p38MAPK.

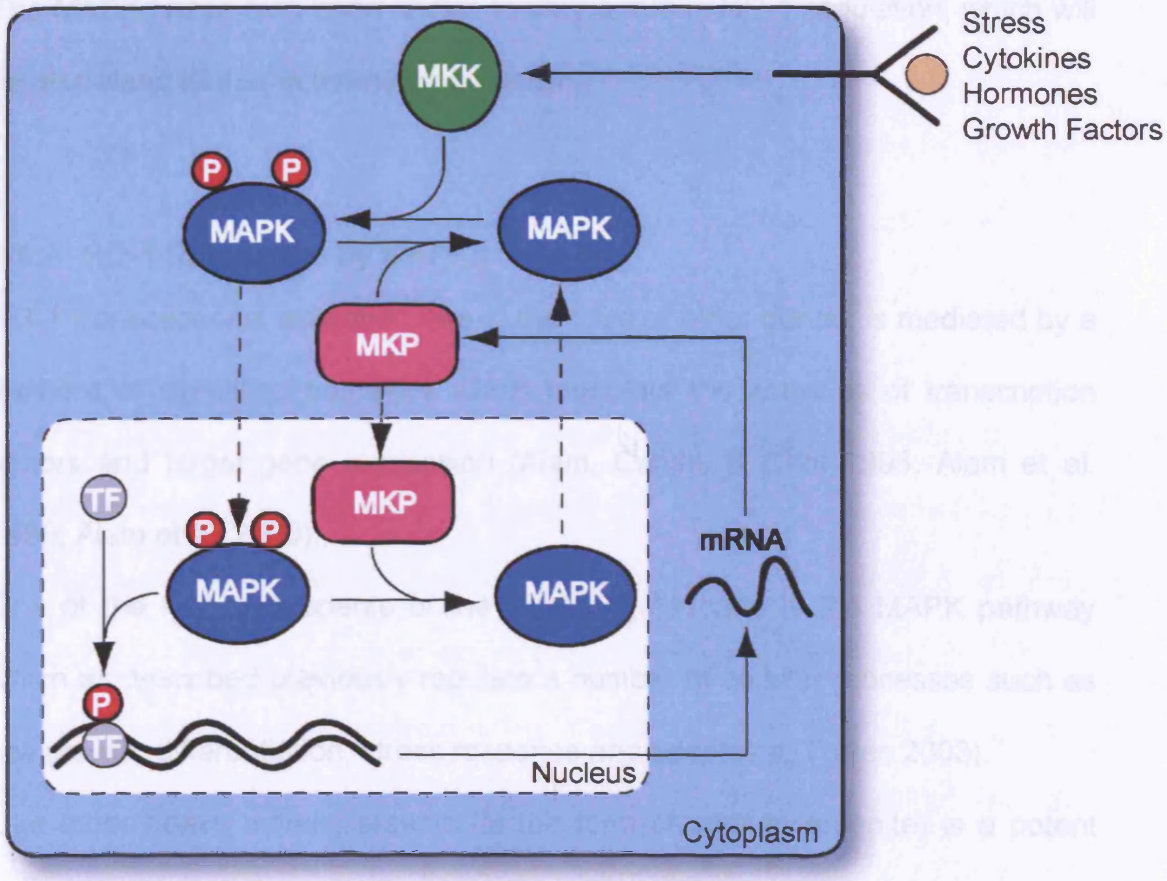


Figure 1.6:- Schematic diagram of the MAPK signalling pathway.
Various stimuli such as stress or cytokines activate MKK which in turn phosphorylates specific tyrosine and threonine residues in MAPK activating it. These activated MAPKs can then phosphorylate cytoplasmic proteins or regulate gene transcription including the MKPs which provide a negative feedback mechanism and inactivate MAPK. Extract from Farooq A, Cell Signal. 2004 Jul;16(7):769-79.

While recent studies have also demonstrated that lipopolysaccharide (LPS) stimulates ROS production in macrophages via a mechanism that is partly dependent on NADPH oxidase. LPS in these cells activated all three forms of MAPK. Production of IL-1 in LPS stimulated macrophages was shown to be dependent on ROS and a PTK/P13K/Rac/p38 pathway. ROS mediated activation of the MAPK pathways plays a significant role in cytokine gene regulation and may act in concert with the NF-κB pathway (Torres 2003).

The MAPKs have also been shown to play a role in HO-1 regulation, which will be discussed further in the next paragraph.

1.5.2 HO-1 Regulation by MAPKs

HO-1 transcriptional activation, like in the case of other genes, is mediated by a plethora of signalling pathways which modulate the activities of transcription factors and target gene expression (Alam, Camhi, & Choi 1995; Alam et al. 1999; Alam et al. 2000).

One of the key components of the signalling cascade is the MAPK pathway which as described previously regulate a number of cellular processes such as cell growth, differentiation, stress response and apoptosis (Torres 2003).

Like other heavy metals, arsenic (in the form of sodium arsenite) is a potent inducer of HO-1. The mechanism of induction seems to be cell and species dependent. Arsenite is known to induce the ERK, JNK and p38 cascade which appears to be mediated by the activation of Shc due to tyrosine phosphorylation and recruitment of the Grb2-Sos complex which can activate the p21 small GTPases of the Rho family Ras and Rac. Ras in turn then activates either the ERK pathway or via action on PAK the JNK or p38 pathway. Rac activation can lead again to PAK activation and subsequently to the activation of JNK and p38. MAPK involvement in HO-1 gene activation in various tumour cell lines seems to be throwing up conflicting data in many studies (Kietzmann, Samoylenko, & Immenschuh 2003).

Kietzmann *et al* demonstrated that kinases of the JNK pathway are involved in the induction of rat HO-1 gene expression by sodium arsenite in primary culture rat hepatocytes (Kietzmann, Samoylenko, & Immenschuh 2003). Rat HO-1

gene expression is induced by Ras via MAPKs through the JNK (MEKK1, JNK) pathway but not via MAPKs of the ERK (Raf, ERK2) pathway. The activation of JNK was mediated by a CRE/AP-1 element which was bound mainly by c-jun. MKK3, which acts as both an inducer of HO-1 and a specific inhibitor of p38MAPK, did not inhibit HO-1 induction by sodium arsenite alone. The MKK3 dependent induction was partially mediated by p38MAPK via an E-box element (-47/-42) which also appeared to be the target for direct inhibition of HO-1 expression by the p38 kinases α, β and δ. The target site for direct inhibition was bound by Max. Overexpression of Max, like p38 α, β and δ isoforms, inhibited HO-1 expression (Kietzmann, Samoylenko, & Immenschuh 2003).

Recently Kacimi *et al* have reported the involvement of p38MAPK in regulation of HO-1 expression under hypoxic conditions. They also observed that SB203580 inhibited hypoxia inducible HO-1 expression in cardiomyocytes whereas PD98059 or tyrosine inhibitors had no effect.

Ryter and colleagues demonstrated that inhibitors of MAPK pathway dependent on p38MAPK and MEK1 activate HO-1 mRNA expression in PAEC and VSMC under hypoxic conditions and to a lesser extent under normoxia (Ryter et al. 2002).

There would also appear to be an intermediary role for MAPK activation during chemical induction of HO-1 gene expression. For example, induction of *ho-1* transcription in MCF-7 cells by cadmium chloride ($CdCl_2$) was blocked by SB203580 but not PD98059. The paradigm from the previous studies implies a general positive correlation between p38MAPK and *ho-1* activation. It is apparent, however, that the regulatory function and relative importance of p38MAPK and MEK1/2-dependent pathways in the regulation of *ho-1* gene

expression may vary significantly in a tissue and inducer specific manner (Ryter, Xi, Hartsfield, & Choi 2002).

Otterbein and co-workers have recently shown that the actions of CO, an important product of HO activity, clearly involve the MAPK signalling cascade (Otterbein et al. 2003a). In particular, they implicate the MKK3/p38 β pathway. They showed that deletion and/or inhibition of the MKK3/p38 β pathway not only modulated the expression of the inflammatory cytokines but also abrogated the CO mediated cytoprotection against oxidant lung injury. The JNK pathway was shown not to be involved in this elicitation of function. Meanwhile, studies have also shown that CO can prevent apoptosis through activation of the p38 pathway (Otterbein, Otterbein, Ifedigbo, Liu, Morse, Fearns, Ulevitch, Knickelbein, Flavell, & Choi 2003a).

An alternate form of HO induction lies in myeloperoxidase, a haem protein involved in host defence.

1.6 Myeloperoxidase

Nearly a century ago the potential biological utility of hypochlorite as a wound disinfectant was described. Some fifty years later Agner identified the 'green haem protein' myeloperoxidase (MPO) as an enzymatic source of chlorinating oxidants (Podrez, Abu-Soud, & Hazen 2000). MPO is a member of the homologous mammalian peroxidase family and is the most abundant protein of neutrophils (polymorphonuclear leukocytes) accounting for approximately 5% of their dry weight. MPO is principally involved in host defence and a means of invoking tissue injury. It does this via the H_2O_2/Cl^- system which generates

HOCl, a potent chlorinating agent, and other chlorinating oxidants (Podrez, Abu-Soud, & Hazen 2000; Carr, McCall, & Frei 2000).

MPO has been found in human and animal atherosclerotic lesions along with ceruloplasmin, lipoxygenase, endothelial NOS (eNOS), and inducible NOS (iNOS) all of which can cause or contribute to LDL oxidation *in vitro* (Carr, McCall, & Frei 2000).

MPO is a tetrameric, heavily glycosylated basic haem protein of ~150 kDa. It is comprised of two identical disulfide linked protomers, each of which possesses a protoporphyrin containg 59-64 kDa heavy subunit and a 14 kDa light subunit (Podrez, Abu-Soud, & Hazen 2000).

The haem protein is secreted from its store, in the primary azurophilic granules of leukocytes, into the extracellular milieu and phagolysomal compartment following phagocyte activation. The activation of phagocyte and excretion of MPO are often accompanied by an oxidative burst generating superoxide (O_2^-) and H_2O_2 via the NADPH complex (Hazen et al. 1999). The oxidising potential of H_2O_2 is amplified by MPO through a peroxidase cycle to generate a host of reactive oxidants and diffusible radical species. MPO is capable of forming multiple intermediary states which are influenced by the availability of reduced oxygen species such as O_2^-, and H_2O_2 and NO. At ground state MPO exists in the ferric (FeIII) form (see Figure 1.7) (Podrez, Abu-Soud, & Hazen 2000; Burner et al. 2000).

The ability of MPO to generate chlorinating oxidants under physiological conditions is a unique and defining activity for the mammalian enzyme.

Substrates for MPO are generally of low molecular weight due to its active site being located at the base of a narrow, deep, hydrophobic pocket. Naturally

occurring substrates for MPO include nitrite (NO_2^-), tyrosine, ascorbate, urate, catecholamines, estrogens, and serotonin. Diffusible oxidant species generated by the substrates can lead to the formation of potent signalling molecules, such as through initiation of lipid peroxidation or xenobiotic intermediate activation (Podrez, Abu-Soud, & Hazen 2000).

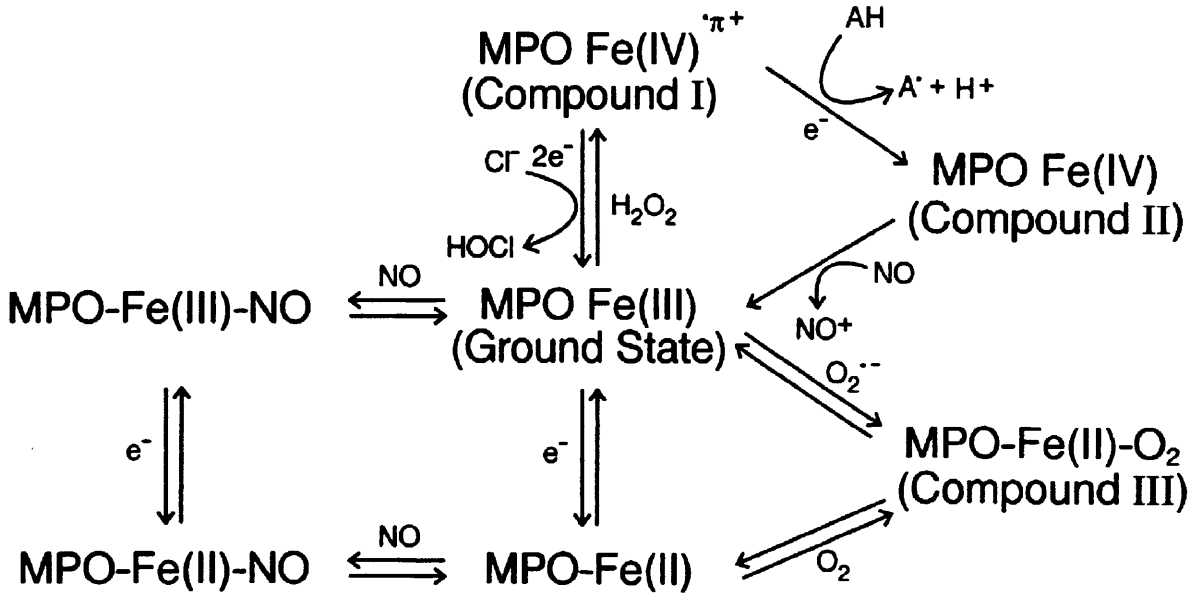


Figure 1.7:- Kinetic model for myeloperoxidase.
An extract from Podrez *et al* Free Radic Biol Med. 2000 Jun 15;28(12):1717-25.

Regulation of MPO activity was thought to primarily rely on the rate of O_2^- production, H_2O_2 availability, and other cosubstrates, or the level of antioxidant species such as ascorbate or methionine. However, recent studies have identified a role for NO in the modulation of MPO activity (Hazen, Zhang, Shen, Wu, Podrez, MacPherson, Schmitt, Mitra, Mukhopadhyay, Chen, Cohen, Hoff, & Abu-Soud 1999). Both NOS and MPO are colocalised in the primary granule of leukocytes. Upon activation of phagocytes both are secreted into the phagolysosome and extracellular compartements where upon nitration of

bacteria proteins occur. Low NO levels are associated with an increase in MPO catalysed peroxidation of substrates. High NO levels, however, result in reversible inhibition of MPO through the formation of an MPO-Fe(III)-NO complex. Furthermore, NO can increase the turnover rate of MPO through the peroxidase cycle by nearly 1000-fold. These interactions of NO and MPO mean that MPO can serve as a catalytic sink for NO, thus limiting its bioavailability, whilst, also detailing just how complex the regulation of MPO is (Hazen, Zhang, Shen, Wu, Podrez, MacPherson, Schmitt, Mitra, Mukhopadhyay, Chen, Cohen, Hoff, & Abu-Soud 1999).

Immunohistochemical studies have demonstrated that nitrotyrosine is enriched in human atherosclerotic intima and LDL recovered from human atheroma (Beckmann et al. 1994). Recent studies have demonstrated that there are two MPO dependent pathways for nitrotyrosine formation via reactive nitrogen species intermediates (see Figure 1.8) (Hazen, Zhang, Shen, Wu, Podrez, MacPherson, Schmitt, Mitra, Mukhopadhyay, Chen, Cohen, Hoff, & Abu-Soud 1999). The first pathway involves MPO dependent oxidation of NO_2^-, an end product of NO metabolism, forming a RNS, whilst, the second pathway involves secondary oxidation of NO_2^- by MPO generated HOCl. Both free and protein bound tyrosine residues can be converted into nitrotyrosine by these pathways. Studies have shown that the MPO-H_2O_2-NO_2^- pathway initiates lipid peroxidation in complex biological media such as human serum. While recent investigations have identified MPO-dependent oxidation of NO_2^- as a primary mechanism for promoting LDL lipid peroxidation (particularly at low rates of NO flux) (Carr, McCall, & Frei 2000).

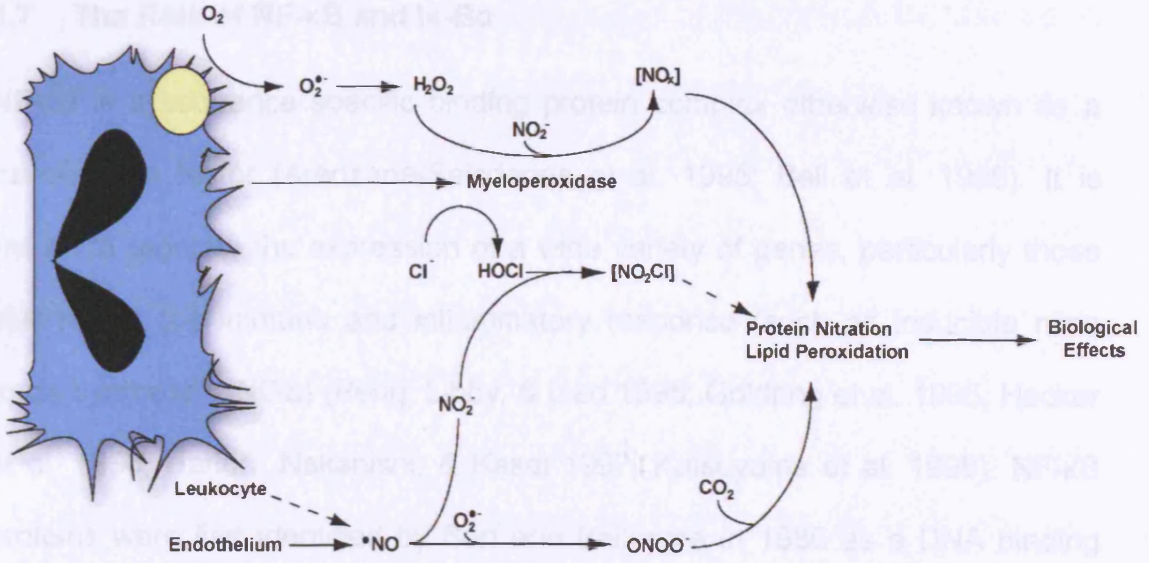


Figure 1.8:- Potential pathways of NO derived oxidant formation by MPO and leukocytes.
Extract from Podrez *et al* Free Radic Biol Med. 2000 Jun 15;28(12):1717-25.

MPO is now established as one mechanism for promoting oxidative modification of proteins and lipids at sites of cardiovascular disease and has a role in immune surveillance and host defence as demonstrated by an enhanced susceptibility to fungal and yeast infection in humans and specific MPO knock out mice (Aratani et al. 2000). It is also tempting to speculate that MPO may have many more biological functions considering its abundance in both neutrophils and monocytes (Babior 2000; Podrez, Abu-Soud, & Hazen 2000; Lefkowitz & Lefkowitz 2001).

1.7 The Role of NF-κB and Iκ-Bα

NF-κB is a sequence specific binding protein complex otherwise known as a transcription factor (Arenzana-Seisdedos et al. 1995; Bell et al. 1996). It is known to regulate the expression of a wide variety of genes, particularly those involved in the immune and inflammatory response (such as inducible nitric oxide synthase - iNOS) (Peng, Libby, & Liao 1995; Goldring et al. 1995; Hecker et al. 1996; Hattori, Nakanishi, & Kasai 1997; Katsuyama et al. 1998). NF-κB proteins were first identified by Sen and Baltimore in 1986 as a DNA binding activity which recognised an 11 base pair sequence (5' GGGGACTTTCC 3') (Matthews & Hay 1995). So far, all NF-κB dependent transcription genes have been shown to share the previously mentioned specific DNA binding motif in their promoters for NF-κB (Peng, Libby, & Liao 1995; Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995).

NF-κB itself is composed of two separate subunits made of different sized polypeptide sequences; the p50 subunit (molecular weight 50,000) and the p65 subunit (molecular weight 65,000) (Peng, Libby, & Liao 1995; Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995; Matthews et al. 1996a). Both belong to the multigene *rel* family of proteins which is composed of proteins involved in the transcriptional regulation of processes such as the expression of inflammatory cytokines (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews et al. 1996b). The *rel* domains present in *rel* proteins regulate gene expression by allowing dimerisation, nuclear translocation and DNA binding (Peng, Libby, & Liao 1995; Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Renard et al. 2000).

Regulation of NF-κB is accomplished by control of the intracellular localisation of the molecule (Peng, Libby, & Liao 1995; Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; de Vera et al. 1997; Jeon et al. 1998; Renard, Percherancier, Kroll, Thomas, Virelizier, Arenzana-Seisdedos, & Bachelerie 2000). In un-stimulated cells NF-κB is retained in the cytoplasm in a non-DNA binding form by IκBα proteins (Peng, Libby, & Liao 1995; Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Renard, Percherancier, Kroll, Thomas, Virelizier, Arenzana-Seisdedos, & Bachelerie 2000). IκBα (also referred to as MAD-3) belongs to a family of inhibitory proteins, including IκBβ, IκBγ, BcL-3 and IκB, all of which are characterised by their ability to bind one or more *rel* family members and to contain multiple ankyrin domains (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995). These ankyrin repeat motifs in IκBα are responsible for the inhibition of the DNA binding activity in *rel* family proteins (Matthews & Hay 1995). NF-κB/*rel* proteins have been shown to share another common feature, this being the presence of a nuclear localisation signal (NLS). The NLS sequence is important in allowing the nuclear uptake of NF-κB/*rel* family proteins and has also been shown to be necessary for IκB/ankyrin repeat motif interactions (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995; de Vera, Taylor, Wang, Shapiro, Billiar, & Geller 1997). Under conditions of no stress (i.e. normality) IκBα is bound to the NLS which has two important effects; as mentioned earlier NF-κB is retained in the cytoplasm due to IκBα masking the

NLS, and secondly it causes NF-κB to undergo an allosteric change preventing it from binding DNA (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995).

In response to an inflammatory stimuli, however, IκB proteins are proteolytically degraded by the 26S multicatalytic proteasome thus freeing NF-κB (see Figure 1.9) (Schini-Kerth et al. 1997; Katsuyama, Shichiri, Marumo, & Hirata 1998;Nomura, Uehara, & Nishiya 1999; Renard, Percherancier, Kroll, Thomas, Virelizier, Arenzana-Seisdedos, & Bachelerie 2000). IκB proteins are targeted for degradation by the phosphorylation of serine residues 32 – 36. Interestingly, it has been suggested that NO may modulate the phosphorylation of IκB. It has been shown that NO can activate protein phosphatases in blood mononuclear cells which leads to the possibility that NO could prevent the release of NF-κB by dephosphorylation of IκB (Peng, Libby, & Liao 1995). Once IκBα is degraded the NLS sequence is unmasked and, therefore, NF-κB is transported to the nucleus where it is able to bind to its specific DNA binding motif and initiate transcription (see Figure 1.10) (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Schini-Kerth, Boese, Busse, Fisslthaler, & Mulsch 1997; Katsuyama, Shichiri, Marumo, & Hirata 1998).

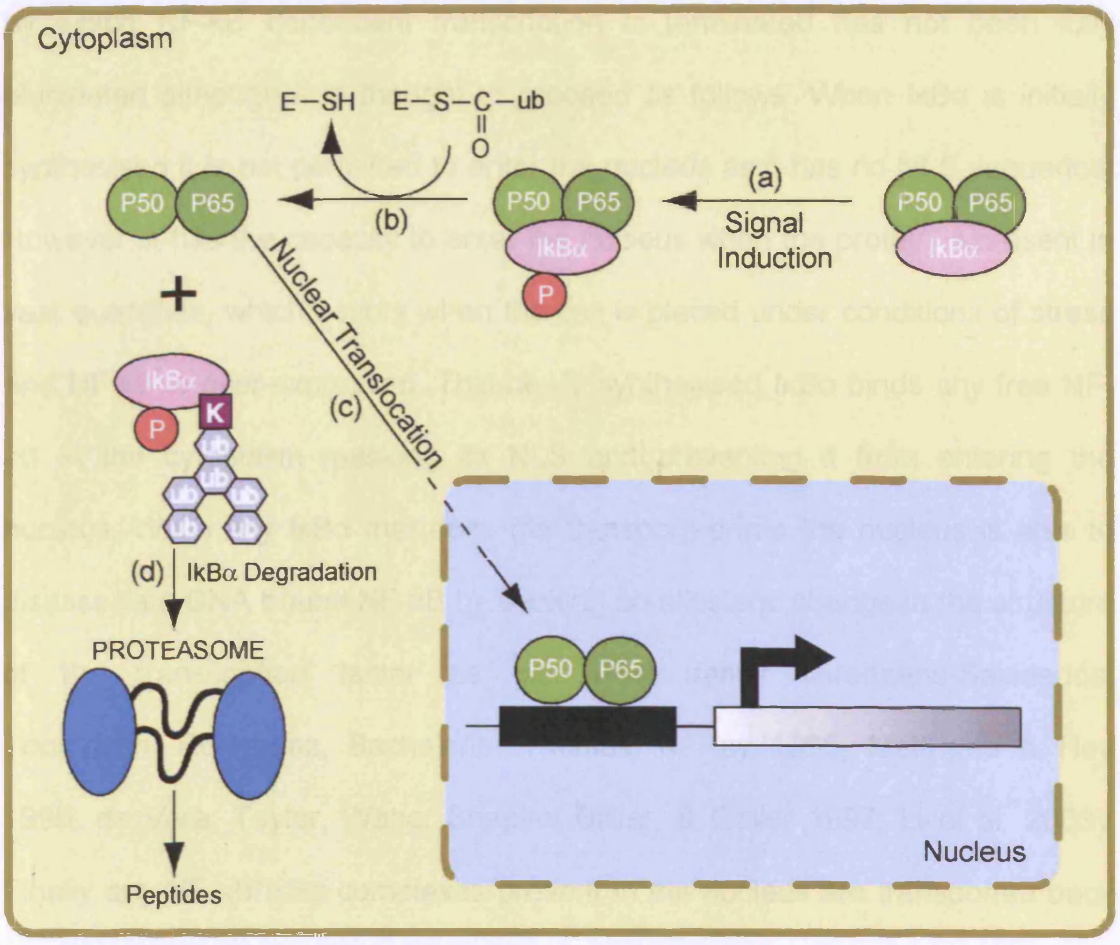


Figure 1.9:- Schematic diagram of IκBα degradation.
(a) IκBα is phosphorylated at residues S32 and S36, after signal induction, converting it into a substrate for a specific ubiquitin ligase. (b) IκBα dissociates from NF-κB when covalently modified by ubiquitin. (c) The free NF-κB is able to translocate to the nucleus. (d) The ubiquinated IκBα is rapidly degraded by the proteasome. Extract from (Matthews & Hay 1995).

As well as transcribing important inflammatory genes, such as iNOS, HIV and IL-2R, NF-κB also binds to IκBα promoters thus increasing the transcription of this inhibitory protein (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995). This results in a massive accumulation of IκBα in the cell which, interestingly, coincides with a loss of NF-κB binding activity and the termination of NF-κB dependent transcription (Renard, Percherancier, Kroll, Thomas, Virelizier, Arenzana-Seisdedos, & Bachelerie 2000). The mechanism

by which NF-κB dependent transcription is terminated has not been fully elucidated although it is thought to proceed as follows. When IκBα is initially synthesised it is not permitted to enter the nucleus as it has no NLS sequence. However, it has the capacity to enter the nucleus when the protein is present in vast quantities, which occurs when the cell is placed under conditions of stress and NF-κB is over-expressed. This newly synthesised IκBα binds any free NF-κB in the cytoplasm masking its NLS and preventing it from entering the nucleus. While any IκBα that does get transported into the nucleus is able to disassociate DNA bound NF-κB by evoking an allosteric change in the structure of the transcription factor as discussed earlier (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews & Hay 1995; de Vera, Taylor, Wang, Shapiro, Billiar, & Geller 1997; Li et al. 2003). Finally any NF-κB/IκBα complexes present in the nucleus are transported back to the cytoplasm as IκBα again masks the NLS sequence, thus ensuring that NF-κB dependent transcription is only transient (Arenzana-Seisdedos, Thompson, Rodriguez, Bachelerie, Thomas, & Hay 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Renard, Percherancier, Kroll, Thomas, Virelizier, Arenzana-Seisdedos, & Bachelerie 2000).

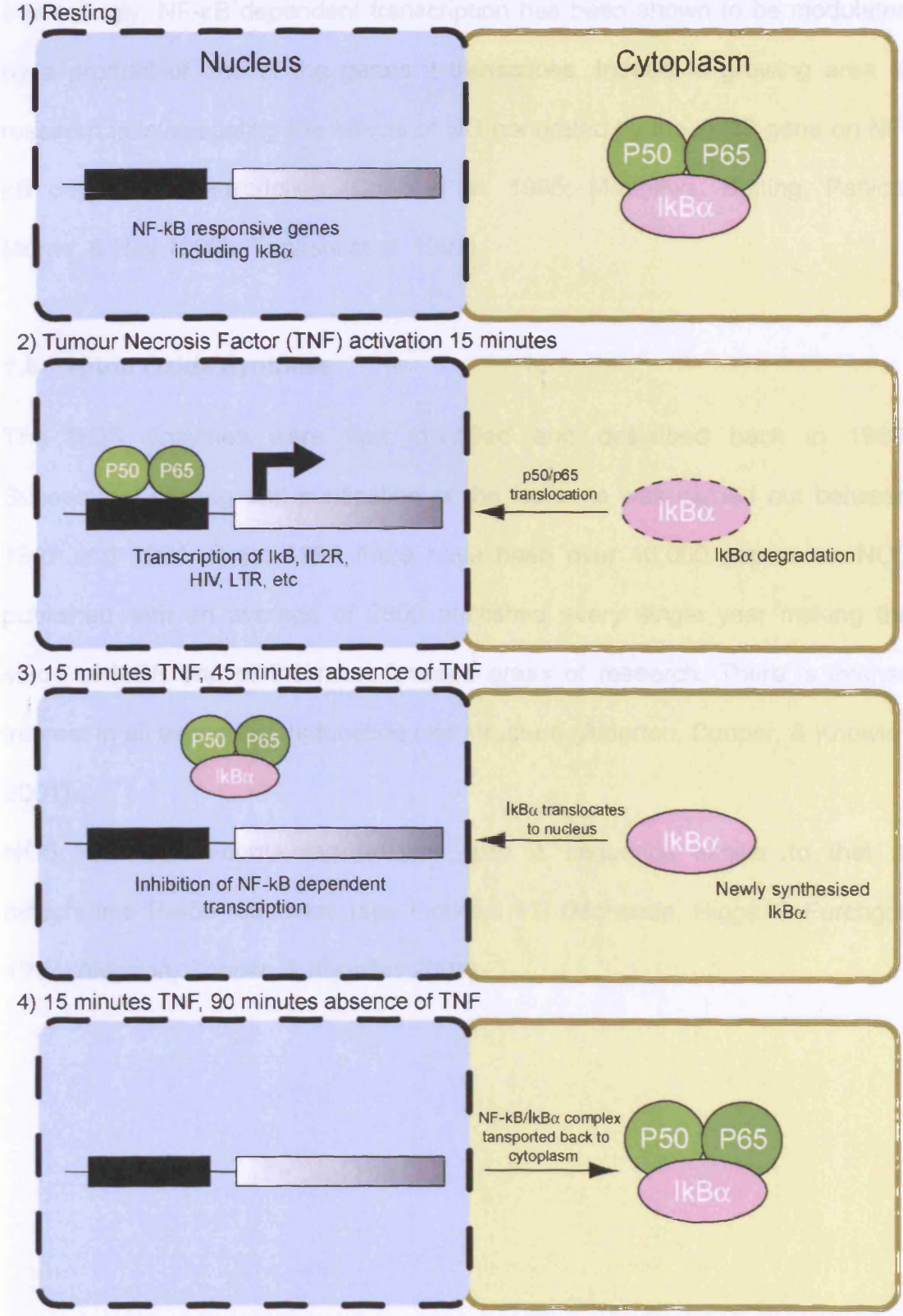


Figure 1.10:- Schematic diagram of NF-κB dependent gene transcription. A proposed mechanism for the re-export of NF-κB - IκBα complexes from the nucleus to the cytoplasm which ensures transient activation and transcription of NF-κB dependent genes. Extract from (Matthews & Hay 1995).

Interestingly, NF-κB dependent transcription has been shown to be modulated by a product of one of the genes it transcribes. Indeed, a growing area of research is investigating the effects of NO generated by the iNOS gene on NF-κB dependent transcription (Chen et al. 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Togashi et al. 1997).

1.8 Nitric Oxide Synthase

The NOS enzymes were first identified and described back in 1989. Subsequent cloning and purification of the isoforms was carried out between 1991 and 1994. Since 1994 there have been over 16,000 papers on NOS published with an average of 2800 published every single year making the study of NOS one of the most focused areas of research. There is intense interest in all aspects of its function and structure (Alderton, Cooper, & Knowles 2001).

NOS is a haem-containing enzyme with a sequence similar to that of cytochrome P-450 reductase (see Figure 1.11) (Moncada, Higgs, & Furchgott 1997; Alderton, Cooper, & Knowles 2001).

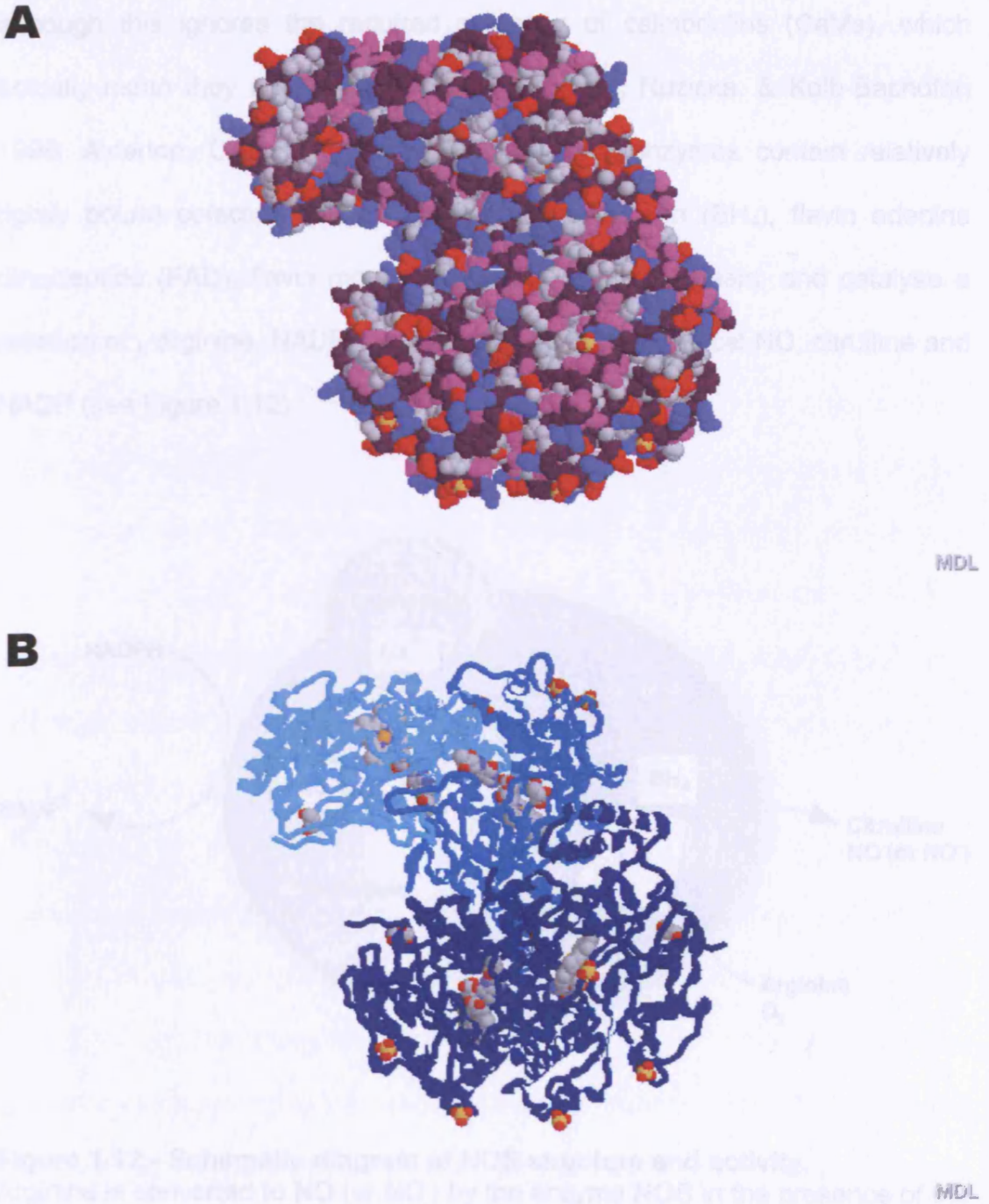


Figure 1.11:- The structure of the human iNOS enzyme.
Diagram (A) displays the protein structure, while diagram (B) reveals the protein motifs present in iNOS. Both diagrams were rendered using Protein Data Bank 3DB Browser, which is available on line at http://pdb-browsers.ebi.ac.uk/pdb-bin/pdbmain.

Generally the NOS enzymes are referred to as dimeric in their active forms although this ignores the required presence of calmodulins (CaMs), which actually mean they are tetramers (Bruch-Gerharz, Ruzicka, & Kolb-Bachofen 1998; Alderton, Cooper, & Knowles 2001). The enzymes contain relatively tightly bound cofactors (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and haem, and catalyse a reaction of $_L$-arginine, NADPH and oxygen to the free radical NO, citrulline and NADP (see Figure 1.12).

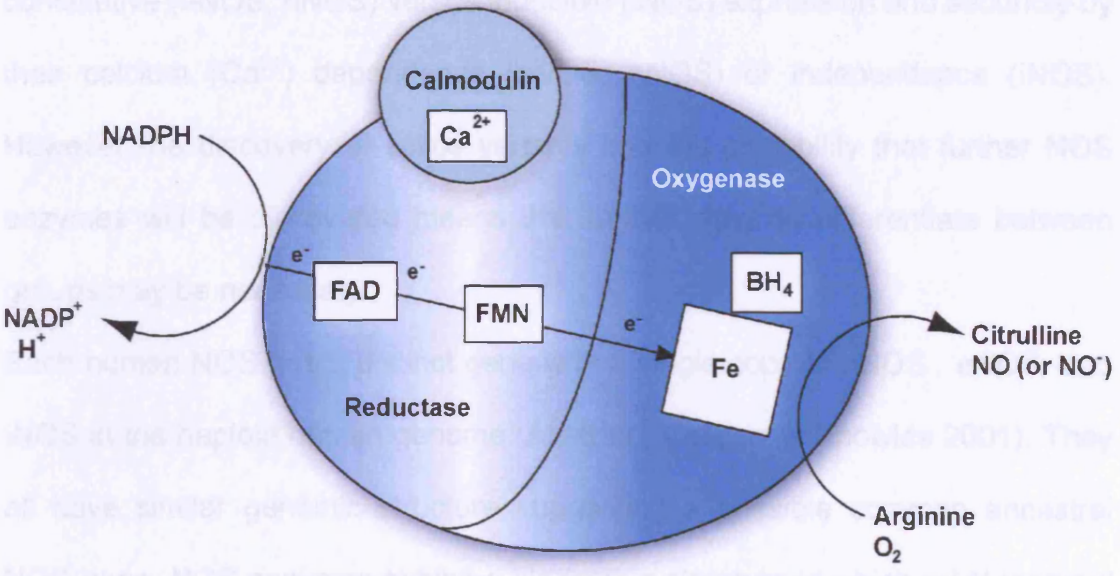


Figure 1.12:- Schematic diagram of NOS structure and activity.
Arginine is converted to NO (or NO^-) by the enzyme NOS in the presence of the cofactors haem, FAD, FMN and BH_4. Extract from Alderton W. *et al*; Biochem J. 2001 Aug 1;357(Pt 3):593-615

There are currently three distinct isoforms of NOS known to exist, each a product of different genes, with different localisation, regulation, catalytic properties and inhibitor sensitivity (Kubes 2000;Alderton, Cooper, & Knowles

2001; Parfenova, Neff, III, Alonso, Shlopov, Jamal, Sarkisova, & Leffler 2001). There is a 51-57% homology between the human isoforms. The three isoforms have been labelled as follows; i) nNOS (also known as Type I, NOS-I and NOS-1) the first identified isoform and mostly found in neuronal tissue; ii) iNOS (also known as Type-II, NOS-II and NOS-2) a highly inducible isoform widely distributed in both cells and tissues; iii) eNOS (Type-III, NOS-III and NOS-3) an isoform first found in vascular endothelial cells (Weinberg et al. 1995; Colleluori, Morris, Jr., & Ash 2001; Alderton, Cooper, & Knowles 2001). The three isoforms have been differentiated in a number of ways. Firstly on the basis of their constitutive (eNOS, nNOS) versus inducible (iNOS) expression and secondly by their calcium (Ca^{2+}) dependence (eNOS, nNOS) or independence (iNOS). However the discovery of splice variants and the possibility that further NOS enzymes will be discovered means that further ways to differentiate between groups may be necessary.

Each human NOS has a distinct gene with a single copy of nNOS , eNOS, and iNOS in the haploid human genome (Alderton, Cooper, & Knowles 2001). They all have similar genomic structure suggesting a possible common ancestral NOS gene. NOS enzymes exhibit a biodomain structure in which an N-terminal oxygenase domain containing binding sites for haem, BH_4 and $_L$-arginine is linked by a CaM recognition site to a C-terminal reductase domain that contains binding sites for FAD, FMN, and NADPH. Both the oxygenase and reductase domain were defined by limited proteolysis. Both domains have been shown to be catalytically active and, interestingly, reconstitution of the second step of NO synthesis has been achieved by the combination of the reductase and oxygenase of human eNOS and murine iNOS respectively.

The human iNOS oxygenase domain (amino acids 82-508) is an elongated shape with a novel α-β fold resembling a baseball mit in which the haem group is located in its palm. The NOS distal pocket is primarily constructed from β sheets which differs considerably from the distal pockets of other haem based oxygenases such a cytochrome P450, peroxidases, and catalases which are all largely α-helical. The two enzymes families appear to have achieved similar catalytic activity through convergent evolution.

iNOS dimer formation is more dependent on the presence of BH_4 than nNOS and eNOS. However, BH_4 as well as haem and $_L$-arginine all promote and/or stabilise the active dimeric form of all three isoforms. The presence of haem appears to be mandatory, with BH_4 and $_L$-arginine promoting dimer formation and stabilising the dimer once formed (Sato et al. 1998). It has also been shown, in the case of iNOS that once the dimer is formed there is little or no significant return to the monomer again in the presence of very potent dimerisation inhibitors. Thus, it is not possible to be sure of the physiological or pathophysiological relevance of monomeric and dimeric NOS distribution (Alderton, Cooper, & Knowles 2001).

NADP and citrulline are both products of NOS activity with N^{ω}-hydroxy-$_L$-arginine (NHA) as an intermediate reaction product. Many authors state $_L$-citrulline as a product but it is not known whether it is $_L$- or $_{D/L}$-citrulline (Bruch-Gerharz, Ruzicka, & Kolb-Bachofen 1998; Alderton, Cooper, & Knowles 2001). Interestingly, the synthesis of NO by NOS is a matter of much debate, whilst, it is clear that cells and tissues containing NOS, and extracts from them, can make NO, this does not necessarily mean that is the initial reactive nitrogen species (RNS) formed, and NO synthesis is often inferred from the

accumulation of breakdown products such a nitrite and nitrate or from reaction with haem proteins such as the oxidation of oxyheamoglobin to methaemoglobin or the stimulation of guanylate cyclase (Alderton, Cooper, & Knowles 2001).

Other potential RNS (e.g. nitroxyl – NO^- or the protonated species HNO, peroxynitrite – $ONOO^-$ ion or the protonated species ONOOH, and nitrosothiols) could in principle be formed first, with the same products and reactions occurring either directly or indirectly after the subsequent reactions to form NO. Indeed, a number of studies have shown interesting results in this area. Hobbs *et al* showed enhanced NO synthesis by iNOS and nNOS in the presence of SOD (Hobbs, Fukuto, & Ignarro 1994). It was concluded that NO^- was being formed and oxidised to NO in a reaction catalysed by SOD, whilst, studies of purified nNOS showed that unless SOD was present they were unable to detect NO formation. This, however, may not be that surprising since SOD can enhance NO formation by removing O_2^- which would otherwise go on to react with NO to from $ONOO^-$. The NO spin trap N-methyl-$_D$-glucamine-dithiocarbamate did detect NO under these conditions leading many to conclude that NO is the major product of NOS, although, this has since been questioned with the suggestion that N-methyl-$_D$-glucamine-dithiocarbamate may also trap NO^-. Interestingly, in the presence of BH_4 and $_L$-arginine substrate, NO formation seems to be favoured, whereas in the absence of BH_4 and NHA as the substrate NO^- is the product. Thus, it seems likely that under different conditions *in vitro* it is possible to get either product. The debate as to which RNS NOS produces is vitally important in understanding both the physiological and pathophysiological implications of NOS and their inhibition. Which may in

part explain the difference between activation of NOS and addition of exogenous NO in so many studies.

Due to the stability of ferrous nitrosyl (Fe^{2+}-NO) complexes, any enzyme that forms a reduced ferrous haem intermediate has the potential to be inhibited by NO. NOS is no exception. For example, the reduction of the suggested Fe^{3+}-NO intermediate would generate Fe^{2+}-NO, as would the reaction of NO^- with the ferric enzyme (Alderton, Cooper, & Knowles 2001). Both iNOS and nNOS can form inhibitory nitrosyl species during turnover. NO inhibition of iNOS appears to be weak and is partially due to the build up of the Fe^{3+}-NO complex; however, up to 95% of nNOS is in the inhibitory Fe^{2+}-NO form seen in the steady state. Unlike iNOS, nNOS also seems to be able to react with NO within the enzyme active site, as the addition of exogenous NO scavengers has little effect on inhibition (Turcanu, Dhouib, & Poindron 1998b). It has been suggested that an Fe^{3+}-NO complex is the final intermediate in the catalytic cycle and that the dissociation of NO from this complex (normal catalysis) competes with its reduction to the Fe^{2+}-NO species (auto inhibition). In general, there are a number of ways to prevent NO inhibition of ferrous haem proteins; i) kinetic prevention of the bond being formed; ii) an increase in the dissociation rate of NO; iii) a modification of the chemical activity of the bond. NOS enzymes generally follow the latter course. The Fe^{2+}-NO bond reacts rather quickly with oxygen generating nitrate and ferric iron. This reaction occurs quickly in iNOS but relatively slowly in nNOS. As such, the rate of oxygen reactions with the nitroxyl complex is one of the steps limiting turnover in the steady state of nNOS. This appears to be due to the presence of a conserved tryptophan residue (Tyr409) in nNOS that hydrogen bonds to the haem thiolate ligand and

increases the stability of the nitroxyl complex thus allowing the enzyme to be controlled by its product.

CaM was the first protein shown to interact with NOS and has been demonstrated to be necessary for the activity of all three isoforms. Both nNOS and eNOS have a much higher Ca^{2+} requirement than iNOS. The binding of CaM increases the rate at which electrons are transferred from NADPH to the reductase domain flavins and artificial electron acceptors such as ferricyanide and cytochrome c; it also triggers electron transfer from the reductase domain to the haem centre. Both nNOS and eNOS have 40-50 amino acid inserts in their FMN binding subdomain, which seems to destabilise CaM binding at low Ca^{2+} concentration and inhibits electron transfer from FMN to haem in the absence of Ca^{2+}/CaM. Phosphorylation of NOS (nNOS and eNOS) has also been shown to have an effect on its activity. Fluid shear stress elicited phosphorylation of eNOS results in an increase in electron flux through the reductase domain and an increase in NO formation (Boo et al. 2002; Iwakiri et al. 2002; Boo & Jo 2003; Davis et al. 2004), whilst, nNOS phosphorylation by CaM-dependent kinases leads to a decrease in NOS activity. There has also been some speculation that an 89 amino acid protein called protein inhibitor of NOS (PIN) could regulate NOS activity, however, it is unclarified and awaits further investigation. As mentioned in paragraph 1.11.3, CO is also capable of binding to the haem of NOS and modulating its activity (White & Marletta 1992; McMillan et al. 1992). Finally, HSP90 has been identified as a regulator of eNOS activity (Shah et al. 1999).

As their abbreviations suggest, eNOS was originally discovered in the vascular endothelium and nNOS in the neuronal tissue, spinal cord and peripheral

nervous system. However, later studies have shown that eNOS is also present in platelets and in certain neuronal populations in the brain, while nNOS has been found in skeletal muscle and in the epithelium of the bronchi and trachea. Also of interest was the discovery that different tissues from the same species contained slightly different forms of the iNOS enzyme (Moncada, Higgs, & Furchgott 1997; Wink et al. 1999). Interestingly, RAW cells and other types of macrophage had been reported to express only iNOS, which generated NO after immunological stimulation. Schmidt *et al;* however, demonstrated a constitutive NOS activity in RAW264.7 macrophages which was found to be as a result of Ca^{2+}-dependent cNOS which was detected in the supernatant and particulate fractions (Schmidt et al. 1992). It was also found that iNOS and cNOS activities in RAW264.7 cells were inversely regulated by LPS and INF-γ, which induced the former and down-regulated the latter.

Other situations known to activate nNOS and eNOS include extreme forms of exercise and pregnancy (Moncada, Higgs, & Furchgott 1997). The iNOS enzyme on the other-hand is activated by a number of factors, which are mainly immunological or inflammatory stimuli such as cytokines (Niemann, Bjorklund, & Eizirik 1994; Moncada, Higgs, & Furchgott 1997; Wink & Mitchell 1998b). However, as with both nNOS and eNOS, iNOS has also been found to break its 'mould'. Indeed, iNOS has been shown to be constitutively expressed in some tissues including; human bronchial epithelium and some fetal tissue (Moncada, Higgs, & Furchgott 1997).

This L-arginine/NO pathway has been shown to be inhibited by several analogues of L-arginine including: N^{ω}-monomethyl-$_L$-arginine (L-NMMA), N^{ω}-nitro-$_L$-arginine methyl ester (L-NAME) and N^{ω}-N^{ω}-dimethyl-$_L$-arginine (ADMA or

L-ADMA) (Moncada, Higgs, & Furchgott 1997; Lancaster, Jr. 1997; Joshi, Ponthier, & Lancaster, Jr. 1999).

1.9 Nitric Oxide

NO is a free radical species which is synthesised intracellulary by the enzyme NOS and has been characterised as the endothelium derived relaxing factor (EDRF) (Ignarro et al. 1987). The metabolism and some of the activities of NO that will be mentioned in this section are highlighted in Figure 1.13.

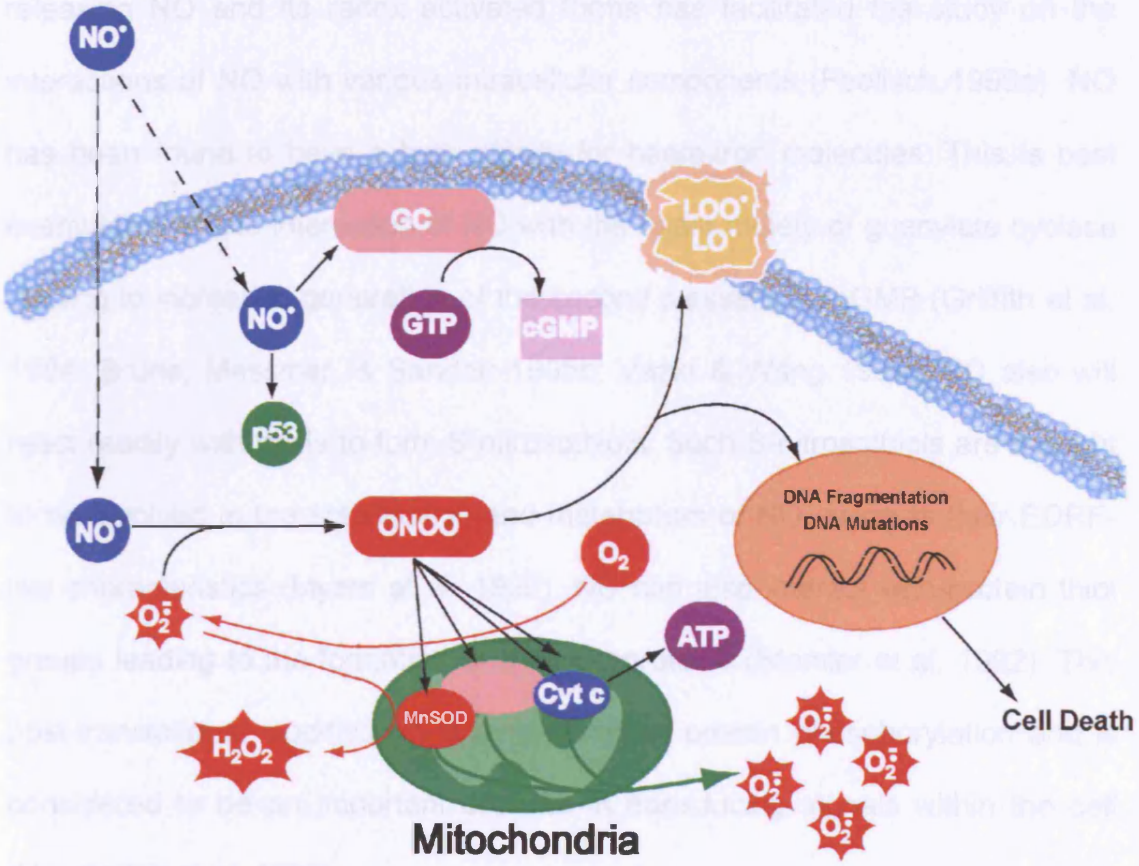


Figure 1.13:- Nitric oxide Metabolism.
This figure illustrates the metabolism of NO and some of the biological activities that NO is involved in. Modified from the Sigma Aldrich Website: http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Signaling/Pathway_Slides_and_Charts/Nitric_Oxide_Metabolism.html

NO is involved in several important physiological processes, which include: control of smooth muscle tone, neurotransmission, inhibition of platelet aggregation and host defence (Moncada, Palmer, & Higgs 1991; Brune, Messmer, & Sandau 1995a).

Recent evidence reveals that the cellular functions of $NO^{\cdot}$ cannot be attributed only to the action of this radical species, but rather to the reactivity of its redox forms. These are specifically, the nitrosonium cation (NO^{+}) and the nitroxyl anion (NO^{-}), the one electron reduction and oxidation products of NO, respectively (Stamler 1994). The use of organic compounds that are capable of releasing NO and its redox activated forms has facilitated the study on the interactions of NO with various intracellular components (Feelisch 1998a). NO has been found to have a high affinity for haem-iron molecules. This is best exemplified by the interaction of NO with the haem moiety of guanylate cyclase leading to increased generation of the second messenger, cGMP (Griffith et al. 1984; Brune, Messmer, & Sandau 1995b; Vaziri & Wang 1999). NO also will react readily with thiols to form S-nitrosothiols. Such S-nitrosothiols are thought to be involved in the stabilisation and metabolism of NO owing to their EDRF-like characteristics (Myers et al. 1990). NO can also interact with protein thiol groups leading to the formation of S-nitrosoproteins (Stamler et al. 1992). This post-translational modification is reminiscent of protein phosphorylation and is considered to be an important process in transducing signals within the cell (Hausladen et al. 1996).

NO has been implicated in many diverse physiological functions including; SMC relaxation, platelet inhibition, neurotransmission, immune regulation and penile erection. The biochemical pathways in these processes share two common

features: the enzymatic synthesis of NO from L-arginine and the formation of an iron nitrosyl complex in target (haem) proteins to evoke the functional response (Murphy & Sies 1991).

The broad chemistry of NO involves many interrelated redox forms: the NO^+, nitric oxide (NO•) and the NO^-. Needless to say there is much disagreement as to the specific generation of each of these forms and the roles they play in the biological system (Feelisch 1998b).

NO has the capacity to modulate the activity of certain proteins through a reversible reaction with the available functional group, most notably Fe and thiols. Guanylyl cyclase activation via the binding of NO to its haem iron is a very important example of this as many cellular responses (vasorelaxation and platelet inhibition) are the result of sGC mediated increases in cGMP (Sogo et al. 2000). The likely method of NO sGC enzyme activation is via an induced structural change, similar to that of O_2 binding haemoglobin. The oxidation state of NO itself and the enzyme critically affect the NO-dependent activation of sGC (Wink & Mitchell 1998a).

The nitrosylation of free thiols has also been shown to modulate specific enzyme activity (Foresti et al. 1997). The fact many diverse proteins of varying function contain critical thiol groups suggest nitrosylation may be involved in broad regulatory actions. The reaction of NO with cell surface thiols for example has been associated with antimicrobial effects, modulation of ligand-gated receptor (NMDA) activity, and alterations of SMC function (Nathan & Hibbs, Jr. 1991; MacMicking, Xie, & Nathan 1997). Signal transduction mediated by adenosine diphosphate (ADP)-ribosylation of protein sulphydryls may be regulated by s-nitrosylation and thus link NO biochemistry to cGMP

independent processes (Ignarro et al. 2001). S-nitrosylation of glyceraldehydes-3-phosphate dehydrogenase has been shown to promote ADP-ribosylation of the enzyme and is associated with the inhibition of its activity. Nitroxyl has also been implicated in the inhibition of sulphydryl dependent enzyme activity (Padgett & Whorton 1995).

The preference of NO for various functional groups is influenced by its redox state. This is best shown by the interaction of haemoglobin and NO•, which reacts with the haem site to inactivate NO, and by way of alternate redox forms of NO at intramolecular amine (NO^+) and sulphydryl (NO^+ and NO–) centres.

Under physiological conditions NO can be interconverted among redox forms which all have distinct chemistries (Stamler, Singel, & Loscalzo 1992).

After release, NO• is susceptible to both oxidation and reduction to NO^+ and NO^- respectively. Both act as primary reaction products that allow secondary target interactions. In biological systems NO can react with O_2, O_2^-, and transition metals forming NO_x, $OONO^-$ and metal-NO adducts respectively (Brune, Messmer, & Sandau 1995b). These reaction products can then go on to react further via redox or additive chemistry interactions. The intracellular prevalence and reactivity of thiols (RSH/RS) over other nucleophiles support nitrosative reactions (NO^+) for s-nitrosothiols (RS-NO) formation. Thus, receptors, ion channels, enzymes or transcription factors containing metals or thiols located at the active or allosteric sites are essential components of NO signaling (Brune, Messmer, & Sandau 1995b).

The interaction of NO with the sGC haem regulatory subunit is a vitally important physiological reaction. The EDRF like function is the key transducer of the vasodilator message from the endothelium to the vascular cells (Wagner

et al. 1997; Hartsfield et al. 1997; Grover et al. 2000). Activation of sGC, formation of cGMP, and concomitant protein phosphorylation forms an inter and intracellular NO responsive regulatory network.

NO cytotoxicity is not only directed against bacteria, microorganisms, and tumour cells as a part of the non-specific immune response but can also affect NO producing cells like macrophages (Schwarz et al. 1995; Brune, Messmer, & Sandau 1995b). NO toxicity has been implicated in major neurodegenerative diseases and pancreatic β-cell destruction linked to type-I diabetes (Gross & Wolin 1995; Mandrup-Poulsen 2001; Boje 2004). NO induced cell death is likely mediated via $OONO^-$ formation, inhibition of FeS enzymes, like the Krebs cycle aconitase, complex I and II of the mitochondrial respiratory chain, or ribonucleotide reductase, deregulation of poly ADP-ribosyltransferase, and energy depletion (Brune, Messmer, & Sandau 1995b). However, NO toxicity depends on the contents of the surrounding biological mileu. NO reactions with reactive oxygen species can have favourable protective outcomes as well as less favourable toxicity. In neuronal cells, the diffusion linked reaction of NO with ROS species results in the generation of toxic $OONO^-$, whereas mesencephalic cells or lung fibroblasts are protected by NO during ischemia reperfusion injury (Brune, Messmer, & Sandau 1995b).

In macrophages (RAW264.7) and β-cell line (RIN m5F), NO-mediated cytotoxicity proceeds through an apoptotic course of cell death and not necrosis. This would appear to be mediated by p53, a tumour suppressor protein that is important in maintaining genetic integrity and has been suggested to act as a 'guardian of the genome' monitoring the state of the cells DNA (Brune, Messmer, & Sandau 1995b).

Recent studies into iNOS expression have indicated that NO may function as a negative feedback modulator of iNOS, thus regulating the production of NO (Buga et al. 1993; Griscavage et al. 1993; Park, Lin, & Murphy 1994; Colasanti et al. 1995; Griscavage, Hobbs, & Ignarro 1995; Weisz, Cicatiello, & Esumi 1996; Matthews, Botting, Panico, Morris, & Hay 1996b; Togashi, Sasaki, Frohman, Taira, Ratan, Dawson, & Dawson 1997; Park, Lin, & Murphy 1997b; Hinz, Brune, & Pahl 2000). A number of mechanisms have been suggested to explain how NO might modulate its own production, many of these focusing on NF-κB activation (Colasanti, Persichini, Menegazzi, Mariotto, Giordano, Caldarera, Sogos, Lauro, & Suzuki 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Park, Lin, & Murphy 1997b; Hinz, Brune, & Pahl 2000). Indeed, several recent studies have shown that exposure of macrophages to NO donors (such as sodium nitroprusside – SNP and S-nitroso-N-acetylpenicillamine - SNAP) leads to inhibition of lipopolysaccharide / tumour necrosis factor α (LPS/TNFα)-elicited NF-κB activation (Colasanti, Persichini, Menegazzi, Mariotto, Giordano, Caldarera, Sogos, Lauro, & Suzuki 1995; Griscavage, Hobbs, & Ignarro 1995; Ma et al. 1996). As NF-κB is the transcription factor involved in iNOS gene transcription, decreasing its activation will likely result in the generation of less NO and, therefore, an eventual increase in NF-κB activation.

NO is thought to regulate NF-κB activation by a variety of mechanisms. Perhaps, the most interesting and important of these is the stabilisation and increased transcription of IκBα (Matthews, Botting, Panico, Morris, & Hay 1996b;Hinz, Brune, & Pahl 2000). As discussed earlier, IκBα is responsible for retaining NF-κB in the cytoplasm and also preventing it from binding DNA thus

inhibiting NF-κB dependent transcription. NO can stabilise IκBα preventing its proteolytic degradation as mentioned in paragraph 1.7 and, therefore, keep it bound to NF-κB (Peng, Libby, & Liao 1995; Griscavage, Wilk, & Ignarro 1996). While activating its transcription increases the level of IκBα present in the cell. This results in IκBα being present in a higher concentration and increases its capacity to bind any free NF-κB in the cytoplasm and facilitates IκBα entering the nucleus to dissociate any DNA bound NF-κB (Matthews, Botting, Panico, Morris, & Hay 1996b; Togashi, Sasaki, Frohman, Taira, Ratan, Dawson, & Dawson 1997). NO is also thought to inhibit NF-κB through S-nitrosylation of the cysteine 62 residue of p50 (Togashi, Sasaki, Frohman, Taira, Ratan, Dawson, & Dawson 1997). The DNA binding activity of NF-κB is located on the p50 subunit and is dependent on the oxidation-reduction state of cysteine on residue 62 (with a reduced form of cysteine required for DNA binding activity). NO might be able to alter these critical thiol groups changing the conformation (and, therefore, the function) thus preventing NF-κB binding DNA (Chen, Kuhn, Sun, Gaydos, & Demers 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Togashi, Sasaki, Frohman, Taira, Ratan, Dawson, & Dawson 1997; Park, Lin, & Murphy 1997a).

It has also been suggested that basal levels of NO are able to keep iNOS expression suppressed by inhibiting NF-κB activation (Rogers & Ignarro 1992; Colasanti, Persichini, Menegazzi, Mariotto, Giordano, Caldarera, Sogos, Lauro, & Suzuki 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Togashi, Sasaki, Frohman, Taira, Ratan, Dawson, & Dawson 1997). This implies that iNOS could be regulated by the endogenous NO generated by constitutive NOS (cNOS) (such as endothelial NOS – eNOS and neuronal NOS – nNOS)

(Colasanti, Persichini, Menegazzi, Mariotto, Giordano, Caldarera, Sogos, Lauro, & Suzuki 1995; Matthews, Botting, Panico, Morris, & Hay 1996b; Togashi, Sasaki, Frohman, Taira, Ratan, Dawson, & Dawson 1997). Assuming that the basal level of NO is kept at a concentration capable of suppressing iNOS expression, then the endogenous NO may contribute to avoid an undesirable and potentially harmful induction of iNOS expression. If on the other hand, the NO level goes below this threshold value, iNOS expression may become easily achievable. In this respect, it seems worthwhile noting that a combination of LPS and interferon γ (IFNγ), which induces the iNOS gene, is also able to simultaneously decrease cNOS mRNA expression in human monocyte/macrophage cells. Furthermore, TNFα has been shown to regulate cNOS by shortening its mRNA half-life in endothelial cells (Colasanti, Persichini, Menegazzi, Mariotto, Giordano, Caldarera, Sogos, Lauro, & Suzuki 1995).

Finally, NO is thought to prevent NF-κB dependent transcription by scavenging the reactive oxygen species (ROS) that are required for NF-κB activation. One such mechanism could involve the activation of the HO pathway (Colasanti, Persichini, Menegazzi, Mariotto, Giordano, Caldarera, Sogos, Lauro, & Suzuki 1995; Motterlini et al. 1996; Turcanu, Dhouib, & Poindron 1998a).

1.10 Interaction of HO and NOS Pathways

Regulation of HO activity and NOS production are intimately linked (Datta, Gross, & Lianos 2002; Johnson et al. 2002; Andre & Felley-Bosco 2003). As well as maintaining/supplementing the cells ability to generate cGMP there is evidence to suggest HO regulates the NOS generation of NO (Datta et al.

1999). This is very important as the dissociation of NO with sGC proceeds very slowly and reversal of NO binding of the haem would not be an effective mechanism for a rapid halt in sGC activation which is crucial for maintaining cellular homeostasis. HO-1 induction can modulate NO production in a number of ways; i) as NOS is a haemoprotein and requires two haems for its active site HO activity would accelerate newly synthesised haem degradation thus impairing de novo synthesis of NOS; ii) due to NOS being a P450 type haemoprotein and cytochromes P450 in intact or denatured form scan substrate for both HO-1 and HO-2 an increased HO activity could accelerate NOS turnover. Cytochrome P450 and HO activity show a reciprocal relationship with HO-1 stimuli causing a concomitant decrease in cytochrome P450; iii) both neuronal and macrophage NOS have been shown to bind CO raising the possibility that HO generated CO could bind and inactivate it; iv) iron released by haem degradation could prevent the nuclear transcription of NOS; v) competition of both HO and NOS for NADPH would favour HO due to its high ratio of concentration plus the need for NADPH in the reduction of biliverdin to bilirubin; vi) HO dependent haem availability could modulate the negative feedback regulation of NO on NOS mRNA expression. NO could also regulate HO activity and CO production by modulating the activity of δ-aminolevulvinate synthase (ALAS), the rate limiting enzyme in haem biosynthesis and the synthesis of the iron storage protein ferritin (Figure 1.14) (Ponka 1999; Taramelli et al. 2000). Iron has been shown to increase HO activity as it downregulates ALAS activity. It is thought iron reguation of ALAS is via iron responsive elements (IRE's) and iron regulatory protein-1 (IRP-1) whilst HO-1 is upregulated via free radical generation. Iron metabolism, in turn, can be

influenced by NO control of IRE binding activity and repression of ferritin synthesis with a possible end result being an increase in HO-1 induction by the free iron made available by a decreased need for the metal in haem biosynthesis and repression of ferritin synthesis.

The interaction of the two systems is present in a number of cell types for example in macrophages where NOS is present in the cytosolic fraction and HO-1 is highly inducible, this form of the oxygenase would become the predominant modulator of NOS activity and NO would activate HO-1 gene expression (Johnson, Teran, Prieto-Carrasquero, & Johnson 2002). As well as its haem/haemoprotein level regulation and generation of biologically active components it is possible that HO (specifically HO-2) could have a role as a haem/oxygen sensor for the cell (Prabhakar 1999). This has been suggested since there is a 100% match in part of its sequence to that of the oxygen consensus sequence and because unlike other haem binding proteins it has two haem regulatory motifs (HRMs) not involved in haem catalysis but in activation of oxygen which could then go on to activate AP-1 and NF-κB two transcription factors activated in the presence of oxygen free radicals. HO-2 could thus function in a regulatory capacity for genes regulated by oxygen radicals, haem, or iron e.g. ferritin, iNOS, and HO-1.

HO may also promote cell injury and killing mediated by the free radical NO•. NO• interaction with oxygen free radicals generated in the course of cellular oxygen metabolism can lead to the formation of peroxynitrite, nitrogen dioxide, and hydroxyl radicals, all of which are more toxic than NO•. The oxygen generating activity of haem bound to HO-2 HRMs as described in the interaction of HO and NOS section via the iron catalysed Fenton reaction could be the

catalyst for the toxic injury to target molecules or cells (Maines 1997; Brune, von Knethen, & Sandau 1998; Jeney et al. 2002). Generation of OH• and superoxide (O_2^-) free radicals is an important mechanism of cytocidal pathways including therapeutic agents, ionising radiation, and toxic chemicals (Brune, von Knethen, & Sandau 1998; Li et al. 2002).

Willis *et al* observed that an increase in HO activity during acute complement development inflammation results in the recruitment of polymorphonuclear (PMN's) cells followed by the later influx of mononuclear (Mns) cells in the pleural activity of carrageenin treated rats. This results in a marked suppression of the inflammatory response. Inhibition of HO activity potentiates this response. This is consistent with the idea of HO-2 generation of oxygen radicals in PMN's and Mns to defend against infection. Further to this is the ability of HO to generate cGMP, produce antioxidants, and deplete haem, which is needed to produce inflammatory arachidonic acid metabolites.

The action of haem can increase bacterial virulence; its binding to receptors on the surface of bacteria is recognised as a virulence marker. Thus degradation of haem by HO could interfere with this and make bacteria avirulent. Regulation of iNOS in such instances of inflammatory response would also play a beneficial and protective role (Genco, Odusanya, & Brown 1994).

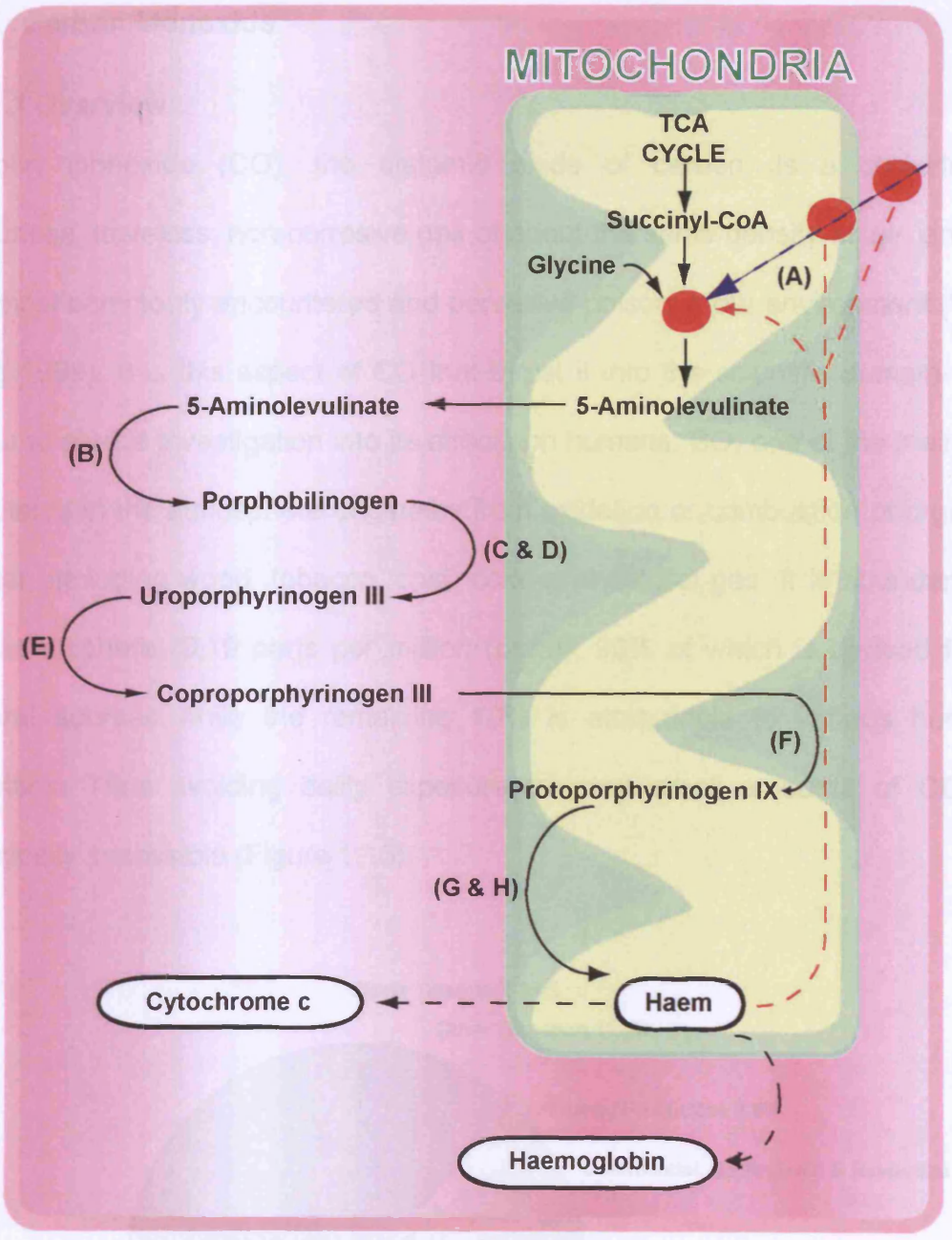


Figure 1.14:- The metabolism of haem in mammals, birds and bacteria.
The enzymes are as follows; (F) = coproporphrinogen oxidase; (G) = protoporphrinogen oxidase; (A) = 5-aminolevulinate synthase; (E) = uroporphyrinogen decarboxylase; (B) = porphobilinogen synthase; (C) = uroporphyrinogen-III synthase; (D) = hydroxymethylbilane synthase; (H) = ferrochelatase.

1.11 Carbon Monoxide

1.11.1 Overview

Carbon monoxide (CO), the diatomic oxide of carbon, is a colourless, odourless, tasteless, non-corrosive gas of about the same density as air, and is the most commonly encountered and pervasive poison in our environment (Von Burg 1999). It is this aspect of CO that thrust it into the scientific domain and ensured a wide investigation into its effects on humans. CO, one of the main air pollutants in the atmosphere originates from oxidation or combustion of organic matter, including wood, tobacco, coal, coke and natural gas. It is abundant in the atmosphere (0.19 parts per million (ppm)), 90% of which is derived from natural sources while the remaining 10% is attributable to various human activities. Thus avoiding dailly exposure to even small amounts of CO is practically impossible (Figure 1.15).

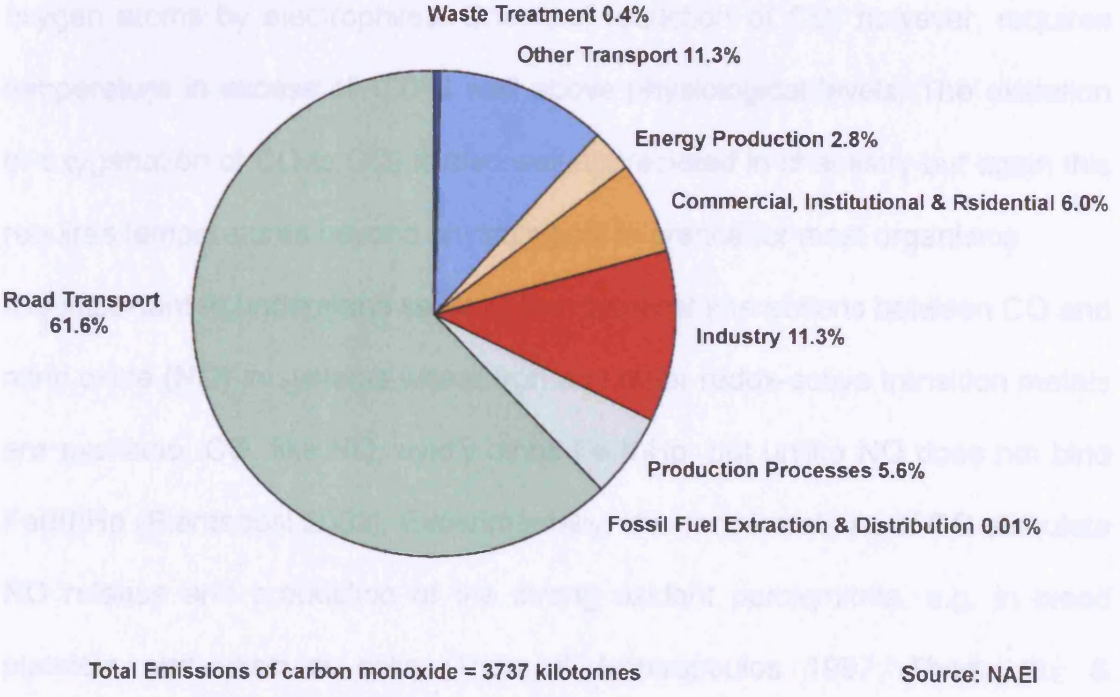


Figure 1.15:- Non-natural carbon monoxide emissions in the UK for 2001. Emissions from human activities. Extract from the environment agency records.

The roles of CO in life may date from the prehistoric environments that were rich in CO, and may be linked to biological pathways that developed during the origins of life. It has recently been proposed that CO made a significant contribution to the synthesis of the first amino and nucleic acids along with oxygen and nitrogen (Kasting 1993).

CO is the anhydride of formic acid; however, it does not react with water without substantial energy input. Although CO is combustible, its formal triple bond makes it chemically quite stable under physiological conditions. Its reaction with molecular oxygen (O_2) is slow and has a high energy of activation; however, CO is involved in redox reactions (Piantadosi 2002). In its free state, CO resists attack by most common reducing agents, including hydrogen (Piantadosi 2002). The reactivity of coordinated CO is much greater than the free gas, and transition metals are particularly effective in promoting reduction of CO. CO readily forms metal carbonyls, which are susceptible to the attack of the CO oxygen atoms by electrophiles. Chemical reduction of CO, however, requires temperature in excess of 100°C well above physiological levels. The oxidation or oxygenation of CO to CO_2 is also well appreciated in chemistry but again this requires temperatures beyond physiological tolerance for most organisms.

It is important to understand some of the chemical interactions between CO and nitric oxide (NO) in systems where iron and other redox-active transition metals are available. CO, like NO, avidly binds Fe(II)Hp, but unlike NO does not bind Fe(III)Hp (Piantadosi 2002). Experimentally, low concentrations of CO stimulate NO release and production of the strong oxidant peroxynitrite, e.g. in blood platelets and vascular cells (Thom & Ischiropoulos 1997; Thom, Xu, & Ischiropoulos 1997b). A chemical mechanism for CO-mediated NO-release has

been proposed based on the redistribution of NO in the cell, a hypothesis consistent with different equilibrium constants for metal binding of the two gases (Olson, Rohlfs, & Gibson 1987; Thom & Ischiropoulos 1997). NO is the most reactive of the physiological gases, having the same effective size and polarity as the O_2 molecule. The rate limiting step for the overall NO binding reaction to haemoproteins (Hp) such as myoglobin is actually NO entry into the binding pocket (Ponka 1999). Thus, the overall association constants for NO with Fe(II)Hp are much faster than the association constants for CO. For haemoglobin, the affinity of NO for Fe(II) is 1,500 times greater than that of CO. The extent to which CO displaces NO from Hp at physiological concentrations of CO, NO and O_2 is unknown. It seems more likely that preformed CO bound to Fe(II), e.g., of cellular Hp, would increase or decrease the bioactivity of newly synthesised NO in response to physiological stimulation or pathological events. This effect would produce differences in the apparent effects of CO as a function of the concentrations and metabolic fates of all three gases. Direct CO-NO interactions will not occur in the met-Hp because CO does not bind to Fe(III) (Sato, Nomura, Sagami, Ito, Daff, & Shimizu 1998). Finally, the interactions among CO, NO and O_2 will be influenced by generation of reactive oxygen species that depend on the presence of reduced transition metals (Maines 1997; Sato, Nomura, Sagami, Ito, Daff, & Shimizu 1998).

Living organisms encounter CO through incidental environmental exposure, usually by inhalation. Cigarette smoke accounts for a major source of CO exposure in humans. CO at certain concentrations, like all agents including life sustaining oxygen, has limits of tolerability. At high concentrations (HbCO level

of 70 % or more), exposure to CO is lethal, usually through accidental smoke or exhaust inhalation in enclosed spaces.

In addition to uptake of exogenous gas, cells and tissues produce significant amounts of CO as an elimination product of cellular metabolism, largely from haem degradation catalysed by HO enzymes. To better appreciate the controversial standing of CO in the scientific community we will review the discoveries that have led to where we are today.

1.11.2 History of Carbon Monoxide

It was first shown that human blood carries a small but measurable CO content over some 50 years ago but the roots of its scientific interest stem from earlier in history (Piantadosi 2002). CO first came to biological attention in 1857 when the French physiologist Claude Bernard determined that the gas produces asphyxia by reversibly combining with haemoglobin (Bernard 1991). In 1895, J.S. Haldane demonstrated that CO binding to haemoglobin could be antagonised by high partial pressures of O_2 (pO_2) and that mammals would survive lethal CO poisoning if a large amount of O_2 was dissolved in blood plasma (Haldane 1895). During World War II, Roughton and Darling reported that carboxyhaemoglobin (HbCO) shifted the oxyhemoglobin dissociation curve to the left because the unoccupied haems of the haemoglobin tetramer bound O_2 with greater affinity after the addition of CO to one site. This effect of CO made it more difficult for the haemoglobin molecule to unload oxygen in the tissues (Roughton & Darling 1944). These pioneering studies gave rise to the common concept of the pathophysiology of CO poisoning based on tissue hypoxia. Accordingly, the decreased arterial O_2 content (CaO_2) in combination

with increased affinity of HbCO for O_2 led to a decrease in tissue PO_2, which produced manifestations of hypoxia in the tissues (Stewart 1975). Although these principles have formed a sound scientific basis for understanding the many biological effects of CO and the treatment of CO poisoning, they do not tell the whole story. Principally, they fail to explain the classical differences between the cellular effects of simple hypoxia and those of CO hypoxia.

In addition to early work on its physiological effects, CO was used as a tool to study tissue respiration. In ground-breaking studies to discover the source of cellular respiration, Otto Warburg, David Keilin, and others, found CO invaluable as a tool to study the biochemical behaviour of intrinsic tissue pigments, identified subsequently as myoglobin and cytochrome c oxidase (cytochrome a,a_3) (Warburg 1930; Keilin & Hartree 1939; Keilin 1966). In the 1920s, Warburg engaged in the study of the role of iron in respiration and found that CO could inhibit respiration in yeast in a light-sensitive manner, extending the 1896 observation of Haldane and Smith, who had discovered that HbCO could be dissociated by exposure to light of appropriate wavelengths (Haldane & Smith 1896). Even now, photodissociation of CO from Hp at picosecond and nanosecond resolution is a powerful approach for studying molecular ligand binding (Olson & Phillips, Jr. 1996). In the 1930s, Fenn and Cobb reported that living tissues burned CO by oxidising it to CO_2; however, mitochondria were not shown to be the source of this oxidising power until almost 50 years later (Fenn 1970).

The many fascinating and curious aspects of CO biology stimulated efforts in the latter half of the 20th century to understand the biological chemistry of CO. In 1952, Sjöstrand and Coburn reported that decomposition of haemoglobin *in vivo*

led to CO generation and that, certain pathological conditions e.g., anaemia could augment it further (SJOSTRAND 1952). Increases in HbCO were attributable to metabolic CO production, particularly after haem degradation had been stimulated by hemolysis (Coburn, Williams, & FORSTER 1964; Coburn, Williams, & Kahn 1966). Specific drugs and chemicals were found to accelerate endogenous CO production, including progesterone. At about the same time Tenhunen *et al* conducted biochemical studies which indicated that endogenous CO was produced primarily from haem catabolism by HO (Tenhunen, Marver, & Schmid 1968). Furthermore, in the microsomal fractions of cells, CO was found to bind to unique cytochromes, now called mixed-function oxidases, and the appearance of the broad CO band of the reduced enzyme in the Soret (UV) region of the spectrum led to the name cytochrome P450 (Estabrook, Franklin, & Hildebrandt 1970).

At the university of Pennsylvania in the 1960s and 1970s, largely through the physiological studies of Coburn, the CO store in the body was measured, and the uptake of CO by tissues, particularly skeletal and cardiac muscle, was shown during ambient exposures. Coburn recognised that the amount of CO in tissues increased substantially during hypoxia due to transfer of the gas from blood to tissue and binding of CO to Hp such as myoglobin (Coburn & Mayers 1971). Similarly, it was demonstrated that CO diffused out of the maternal circulation across the placenta and bound to foetal haemoglobin *in vivo* (Hill et al. 1977).

Within the last 25 years, it has been documented that CO is oxidised in the body to CO_2 by mitochondria (Young & Caughey 1986; Young & Caughey 1990). CO alters the mitochondrial redox state and energy provision in the

brain; these effects persist after HbCO has been cleared from the blood (Brown & Piantadosi 1992). By activating guanylate cyclase, CO inhibits platelet aggregation and acts as a direct vasodilator (Brune & Ullrich 1987). Toxic and presumed subtoxic CO exposure are associated with significant oxidative and nitrosative stress (Thom 1990; Thom, Xu, & Ischiropoulos 1997a; Thom et al. 1999a). It was not until 1991 when Marks *et al* published a paper entitled "Does carbon monoxide have a physiological function?" that the idea of CO as something more than a waste product was considered (Marks et al. 1991). The reasoning behind this idea was that CO could perhaps play a similar role to NO in cell function, regulation and communication due to their similar chemistry. The findings that both biliverdin and bilirubin, two other long considered waste products of haem catabolism, had beneficial properties added credence to these new claims (Stocker et al. 1987; Stocker, Glazer, & Ames 1987; Stocker 1990). As time passed and the number of publications in favour of CO as beneficial end product of haem catabolism grew, the initial scepticism that a long regarded toxic gas could have an important role to play physiologically subsided.

Finally, over the past decade, endogenous CO production has been associated with changes in intra and intercellular signalling processes, e.g., neurotransmission and vasodilation by activating guanylate cyclase (Verma et al. 1993; Maines 1997). Some of these effects of CO appear to be closely related to those of NO whereas others may be direct influences of CO on the behaviour of NO through interactions with iron or other metals. The effects of CO are proving to be important in regulating inflammation, cell death, and cell

proliferation *in vivo*, although the links to specific biochemical reaction in the cell still await elucidation.

Since its discovery as an endogenously formed product of haem degradation the number of biological activities associated with CO has been steadily increasing.

1.11.3 Carbon Monoxide Bioactivity

The release of CO from vascular cells may have important physiological and/or pathophysiological consequences. Under normal conditions, endogenous CO binds to red blood cells (RBC) forming HbCO and is transported to the lungs (Goldbaum, Orellano, & Dergal 1976). The HbCO is equilibrated with inhaled oxygen within the lungs and CO expelled from the body via exhalation, although approximately 1% is retained (see Figure 1.16).

In healthy blood vessels, the basal release of CO by endothelial cells, primarily via the constitutive HO-2 enzyme, may exert important paracrine effects on the underlying smooth muscle cells (SMC) and on circulating blood cells (Durante & Schafer 1998; Dulak et al. 2002). CO has been shown to maintain vascular tone in large and small arteries and directly relax vascular smooth muscle in a reversible manner (Durante & Schafer 1998; Kaide et al. 2001). A threshold concentration of 1 μM CO was sufficient to induce these effects and was not dependent on the presence of an intact endothelium unlike NO (Maines 1997).

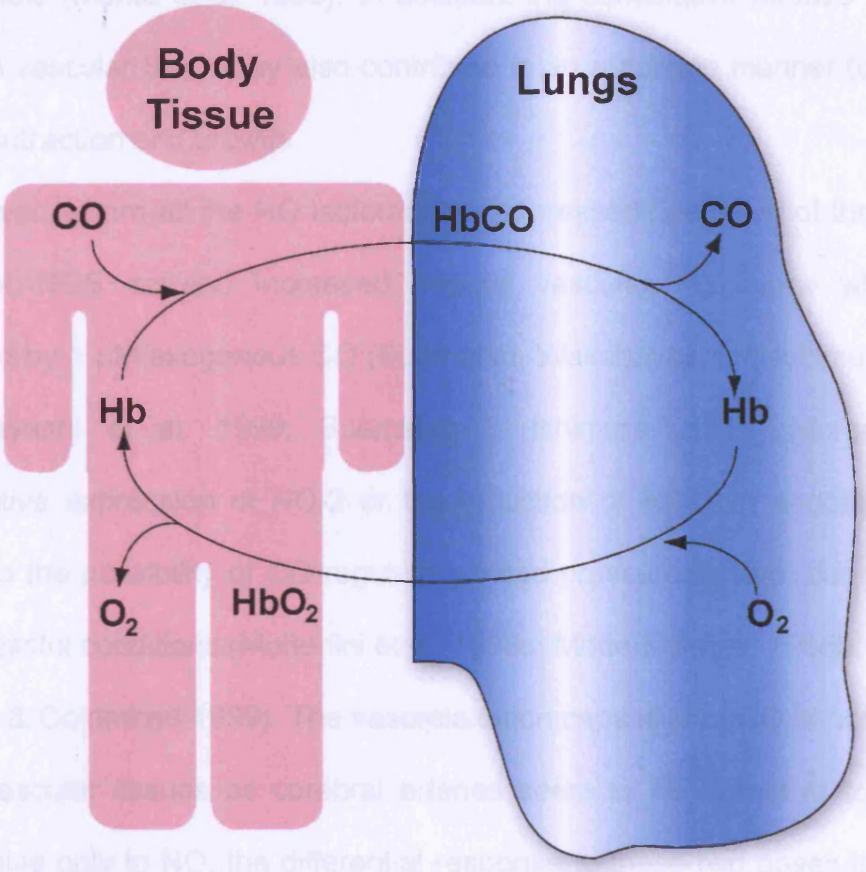


Figure 1.16:- Gas exchange in the body.
CO generated in the tissues diffuses to blood vessels and binds to haemoglobin (Hb) which transports it to the lungs to be expelled where upon the Hb takes up oxygen to deliver to the tissues and the process starts again.

The effects of CO on blood pressure may be regulated directly through the promotion of vasodilation or indirectly via acting on the nucleus tractus solitaius in the brain, which leads to an alteration of glutamatergic transmission and thus a lowering of blood pressure (Maines 1997; Johnson, Kozma, & Colombari 1999;Morse & Sethi 2002). The ablumenal release of CO by endothelial cells may play an important fundamental role in regulating blood flow by inhibiting smooth muscle tone and SMC proliferation, while CO released into the vessel

lumen may modulate blood fluidity by preventing the aggregation and adhesion of platelets (Morita et al. 1995). In addition, the constitutive release of CO by HO-2 in vascular SMC may also contribute in an autocrine manner to regulate SMC contraction and growth.

In the liver, where all the HO isoforms are expressed, inhibition of their activity (but not NOS activity) increased hepatic vascular resistance which was reversed by 1 μM exogenous CO (Suematsu, Wakabayashi, & Ishimura 1996a; Wakabayashi et al. 1999; Suematsu & Ishimura 2000). Moreover, the constitutive expression of HO-2 or the induction of HO-1 in endothelial cells points to the possibility of CO regulating blood pressure *in vivo*, during normal and stressful conditions (Motterlini et al. 1998a; Motterlini et al. 1998b; Johnson, Kozma, & Colombari 1999). The vasorelaxation capability of CO is not common to all vascular tissues as cerebral arteries seem to be refractive to CO, but responsive only to NO; the differential response to these two gases in cerebral arteries is not entirely clear at present. The vascular effects of CO are likely mediated by the activation of soluble guanylate cyclase and the consequent rise in intracellular cGMP (Durante et al. 1997). In this respect, the physiological roles of CO parallel those of NO, and thus these two diatomic gases may exert constitutively additive or synergistic actions on the maintenance of vascular tone and blood fluidity. However, at sites of vascular injury, where endothelial cells are lost or damaged, vascular SMC may be capable of subsuming some of the vasodilatory, anti-proliferative, and anti-thrombotic properties of the endothelium by upregulating CO production via the inducible HO-1 enzyme (Stanford et al. 2003). Following local injury of the blood vessel wall, circulating monocytes and macrophages are recruited to the site of injury, where they are

activated and release various inflammatory cytokines, including Il-1β and TNF-α (Ndisang et al. 2002; Morisaki et al. 2002). These inflammatory mediators can further stimulate the production of cytokines by the blood vessel wall leading to locally elevated levels of cytokines and the induction of HO-1. CO-mediated inhibition of platelet activation and neutrophil migration are likely stimulated by an increasing concentration of cGMP. These characteristics have been suggested as a mechanism by which CO promotes survival of transplanted organs as expression of HO-1 has been shown to protect against chronic rejection (Luckraz et al. 2001; Nakao et al. 2003b). Furthermore, a direct link between CO formation and suppression of xenograft rejection in mouse-to-rat cardiac and liver transplants models was demonstrated by Soares and co-workers, as inhibition of HO-1 induction with tin protoporphyrin promoted an acute rejection episode, whereas exogenous CO restored long-term xenograft survival (Sato et al. 2001). A number of publications have highlighted the anti-inflammatory ability of HO-1 to suppress endothelial cell apoptosis via activation of the p38 mitogen activated protein kinase (MAPK) signal transduction pathway (Brouard et al. 2000; Soares et al. 2002). Moreover, at sites of endothelial loss, vascular SMC may be directly exposed to arterial levels of fluid shear stress, which acts as a potent stimulus for HO-1 expression. Based on the finding that haemodynamic forces selectively induce HO but not NOS in vascular SMC, CO (but not NO) is likely to be the principle mediator of flow-dependant changes in the vascular tone at sites of blood vessel damage (Wagner, Durante, Christodoulides, Hellums, & Schafer 1997). The focal induction of HO-1 and CO release by SMC at sites of vascular injury may promote vascular homeostasis by preventing blood vessel spasm and SMC

proliferation (Otterbein et al. 2003d). SMC can also inhibit platelet aggregation via HO-1 catalysed release of CO, suggesting that SMC derived CO may play an important role in regulating thrombus formation *in vivo*, at sites of injury (Durante & Schafer 1998).

The induction of HO-1 and the generation of CO may also be of pathophysiological significance. Ischemia-reperfusion results in HO-1 induction in both the kidney and the heart (Maines 1997; Zhang et al. 2003). Under these conditions, the induction of HO-1 may serve as an important cellular defence mechanism by enhancing the degradation of the pro-oxidant molecule, haem. Induction of HO-1 also results in the generation of bilirubin and biliverdin as discussed earlier. Both these haem metabolites are efficient scavengers of reactive oxygen species and inhibit lipid peroxidation (Neuzil & Stocker 1994; Kranc et al. 2000). HO-1 mediated production of CO also improves blood supply to these organs by dilating the blood vessels. Similarly, the induction of HO-1 expression following a subarachnoid haemorrhage may serve to increase haem clearance, and enhance the production of anti-oxidant bile pigments as well as vasorelaxant CO, thus reducing the damage resulting from lipid peroxidation and vasospasm (Koehler & Traystman 2002; Otterbein, Soares, Yamashita, & Bach 2003b).

The capacity of NO to induce HO-1 activity in vascular cells may also be of pathophysiological relevance. In response to blood vessel injury, vascular cells express inducible NOS (iNOS) generating copious amounts of NO that could be cytotoxic. The release of CO following HO activation may provide an important mechanism in limiting NO release since CO directly inhibits NOS activity by binding to the haem moiety of the enzyme (Stone & Marletta 1994; Foresti &

Motterlini 1999). In addition, because haem is essential for both the intracellular assembly of the active dimeric NOS and its catalytic activity, increases in HO activity may reduce intracellular haem levels, thereby limiting the formation of active NOS enzyme. Finally, iron released during the course of haem degradation could further inhibit NOS synthesis by inhibiting its nuclear transcription (Wolff et al. 1996). Thus NO induced HO-1 induction may provide an important negative feedback mechanism in limiting the release of cytotoxic levels of NO by vascular cells while still preserving blood flow at sites of vascular injury by releasing CO.

The discovery of a neuronal role for NO led to the suggestion that CO may function as a neurotransmitter, intracellular messenger, and modulator of neuroendocrine function (Marks, Brien, Nakatsu, & McLaughlin 1991; Prabhakar 1998; Baranano & Snyder 2001; Dulak & Jozkowicz 2003). The discovery of HO isoforms, particularly HO-2 throughout the central nervous system (CNS) and peripheral nervous system (PNS) has added weight to the hypothesis that CO is an important gaseous neural messenger, while in certain cases CO formation may substitute for decreased or absent NOS activity (Morse, Sethi, & Choi 2002). In the digestive system, mice lacking HO-2 have an impaired nonadrenergic, noncholinergic (NANC) relaxation response in their mysenteric plexus, a phenotype that was also observed in mice lacking NOS (Chakder et al. 2000; Boehning et al. 2003). In another study, it was reported that CO and NO act as coneurotransmitters in the modulation of neuronal activity in the enteric nervous system (Baranano & Snyder 2001). These examples illustrate the neurotransmitter capacity of CO and support the possibility that CO and NO act as coneurotransmitters in the same neurons.

Certain neurodegenerative disorders, e.g., Alzheimer's and Parkinson's disease sufferers (AD and PD respectively), show an increased expression of HO-1 protein in regions with senile plaques and neurofibrillary tangles, while amyloid precursor protein, which is postulated to be a major cause in the neurotxicity of AD, leads to inhibition of HO (Frankel, Mehindate, & Schipper 2000; Baranano & Snyder 2001). The precise function and mechanism of haem oxygenase and, therefore, CO in these and other neurodegenerative disorders is unclear at present. Another possible neurological function for CO is in the area of learning and memory as there is evidence to support a role for the molecule in long-term potentiation (Zhuo et al. 1993; Piantadosi et al. 1997).

Carbon monoxide also seems to play a role in various aspects of reproduction. During pregnancy, CO maintains normal vascular tone to protect against preeclampsia and relaxes smooth muscle in the uterus (Kreiser et al. 2004). In the placenta, CO has been muted to control functional and developmental maturity as haem oxygenase isoforms are widely distributed in the placenta at different periods of gestation (McLaughlin et al. 2001; Lyall 2003). The reproductive effects of CO are conveyed through the regulation of the endocrine system. For example, CO inhibits the release of corticotrophin-releasing hormone (CRH), arginine vasopressin, and oxytocin, but promotes the release of luteinising hormone-releasing hormone, all of which are involved in the hypothalamus-pituitary-adrenal axis control of pregnancy (Mancuso et al. 1998). Within the male reproductive track, CO originating from specific neuronal populations within the vas deferens may act as a neurotransmitter in the regulation of ejaculation as mice with a targeted deletion of HO-2 exhibit ejaculatory abnormalities (Snyder, Jaffrey, & Zakhary 1998; Burnett et al. 1998).

Secondly, cholinergic nerves which innervate the corpus cavernosum (CC) and spongiosum (CS) contain haem oxygenase isoforms, which suggests a role for CO in penile erection (Ushiyama et al. 2004).

While the local induction of HO-1 and CO production by vascular cells following vascular injury may serve an important protective role in maintaining blood flow and limiting oxidative damage, a more generalised induction of CO synthesis may have deleterious effects. In this respect, a recent study suggests that the widespread induction of HO-1 in the vasculature and the subsequent production of large amounts of CO may contribute to the severe hypotension associated with endotoxin shock (Penney 1988). In addition, high levels of CO can directly damage vascular cells by generating reactive oxygen species. CO binds to mitochondrial haem proteins and disrupts mitochondrial electron transport leading to the release of superoxide anions. High CO exposure also competitively discharges NO from haem proteins which subsequently reacts with superoxide to yield cytotoxic peroxynitrite (Thom, Xu, & Ischiropoulos 1997b; Thom et al. 1999b). Thus, depending on the amounts generated CO can exert both beneficial and harmful effects on the vascular system. Pathological excess of CO production results in a range of toxic effects, the earliest detectable difference being an increase in HbCO levels (10-20%), which due to the high affinity of Hb for CO (~240 times greater than oxygen) interferes with oxygen delivery. CO levels of 40-60% generally result in death due to the binding of CO with other haemoproteins e.g., CYP-450, myoglobin (see Table 1.2).

Measurement of CO in the blood (HbCO) and breath lends itself well to use as an indicator of haem degradation and bilirubin formation as it is expelled

primarily via the lungs and is derived from HO in a 1:1 ratio with bilirubin. Excess HbCO in the blood gives a rosy hue to the complexion of the sufferer much the same as bilirubin excess causes a yellowing of the skin.

In adults, CO is formed at a rate of 0.4 ml/h (16.4 µmol/h). The rate of CO production (V_{co}) is normally relatively constant in males; however, both newborns and females exhibit variations. Under pathological conditions the V_{co} can increase in an adult male from 18 to 160 µmol/h. The effects of which are detailed in Table 1.3 (Stevenson, Vreman, Wong, & Contag 2001).

Table 1.2:- The symptoms and effects of various carboxyhaemoglobin levels in man.

HbCO Level	Signs and Symptoms
0%	Usually none
10%	Frontal headache
20%	Throbbing headache, dyspnea with exertion
30%	Impaired judgment, nausea, dizziness, visual disturbance, fatigue
40%	Confusion, syncope
50%	Coma, seizures
60%	Hypotension, respiratory failure
70%	Death
Reisdorff EJ, Wiegenstein JG. Carbon monoxide poisoning. In: Tintinalli JE, Krome RL, Ruiz E. Emergency Medicine: A Comprehensive Study Guide. 3rd Ed. New York: McGraw Hill; 1992:704.	

Approximately 86% of endogenous CO production is derived from the result of HO action. The remaining 14% is attributable to non-enzymatic sources which include; lipid peroxidation, photo-oxidation of natural or synthetic

photosensitisers e.g., riboflavin, bilirubin and some metalloporphyrins and intestinal bacteria (Maines 1997; Archakov et al. 2002).

Table 1.3:- Effects of excess CO on the human body.

Neuropsychiatric
Coma seizures, agitation, leukoencephalopathy, cerebral edema, behavioral disorders. decreased cognitive ability, Tourette-like syndrome, mutism, fecal and urinary incontinence, parietal lobe dysfunction, ataxia, muscular rigidity, Parkinsonism, peripheral neuropathy, psychosis, memory impairment, gait disturbance, abnormal EEG, personality changes.
Cardiovascular
Angina, tachycardia, ST-segment changes, hypotension, arrhythmias, myocardial infarction, heart block.
Pulmonary
Pulmonary edema and hemorrhage, unilateral diaphragmatic paralysis.
Ophthalmologic
Flame-shaped retinal hemorrhages, decreased light sensitivity, decreased visual acuity, cortical blindness, retrobulbar neuritis, papilledema, paracentral scotomas.
Vestibular and Auditory
Central hearing loss, tinnitus, vertigo, auditory nystagmus.
Gastrointestinal
Vomiting, diarrhea, hepatic necrosis, hematochezia, melena.
Dermatologic
Bullae, alopecia, sweat gland necrosis, "cherry-red" skin color (rare), edema, cyanosis, pallor, erythematous patches.
Musculoskeletal
Rhabdomyolysis, myonecrosis, compartment syndrome.
Renal
Acute renal failure secondary to myoglobinuria, proteinuria.
Metabolic
Lactic acidosis, non-pancreatic hyperamylasemia, diabetes insipidus, hyperglycemia, hypocalcemia, polycythemia.
Foetal
Death, cerebral atrophy, microcephalus, low birth weight, psychomotor retardation, seizures, spasticity.
Reisdorff EJ, Wiegenstein JG. Carbon monoxide poisoning. In: Tintinalli JE, Krome RL, Ruiz E. Emergency Medicine: A Comprehensive Study Guide. 3rd edition. New York: McGraw Hill; 1992:704.

The discovery that CO can bind to the iron within the haem moiety of sGC, thus activating the enzymatic conversion of GTP to cGMP, suggests that CO mediates its biological effects via a cGMP pathway (Durante & Schafer 1998;

Steiner & Branco 2001). This mechanism of activating sGC is similar to that elicited by NO but differs with respect to the bonds formed and intensity of upregulation of the enzyme. Furthermore, the benzylindazole derivative YC-1 can enhance the activation of sGC by CO > 4000%, suggesting an endogenous 'YC-1 like' molecule may enable a higher degree of activation of sGC by CO akin to NO (Friebe & Koesling 1998; Friebe, Mullershausen, Smolenski, Walter, Schultz, & Koesling 1998). Although many studies of CO function have employed metalloporphyrins (e.g. tin protoporphyrin) as inhibitors of haem oxygenase the use of such agents has been questioned due to the possible inhibition of sGC and modulation of NOS activity (Grundemar & Ny 1997).

There is still a great deal about CO to be discovered and as Barinaga commented in Science in 1993, "... this gas is likely to provide fuel to run plenty of labs" (Barinaga 1993).

1.11.4 The Development of Carbon Monoxide-Releasing Molecules

The importance of CO in the biological environment is strongly emerging. Consistent findings have revealed a series of important cellular functions that support a versatile and previously unidentified role for CO gas. It is interesting that many of the novel properties pertaining to CO have strong analogies with the well-established biological activities elicited by NO. Research in the field of NO has been largely facilitated by the development of a variety of organic compounds that spontaneously release NO and can reproduce a physiological or pathophysiological function of NO; however, no attempts have been made to identify or develop similar compounds capable of delivering CO.

The idea that CO could be delivered into the body to target physiological or pathophysiological problems such as the highly successful NO donors is an intriguing one. Such a compound could also be used as a research tool to aid in the elucidation of further functions of CO *in vivo* and *in vitro*. Without the existence of this compound researchers have tried using inhalation of CO gas as an internalisation method, although, the results have been promising the delivery method is not targeted and lacks any real control of how much CO is actually being delivered to the site where upon it is required to act. Not to mention the systemic effect of CO gas on oxygen transport and delivery.

During the past few years our laboratory has been working on the characterisation of biological activities associated with transition metal carbonyls as potential CO releasing molecules (CO-RMs) (Motterlini et al. 2001; Motterlini, Foresti, & Green 2002; Mann & Motterlini 2002; Motterlini et al. 2002a; Johnson et al. 2003; Boehning, Moon, Sharma, Hurt, Hester, Ronnett, Shugar, & Snyder 2003; Motterlini et al. 2003b; Clark et al. 2003b; Foresti et al. 2004). These complexes are compounds that contain a transition metal e.g., iron, manganese and are surrounded by carbonyl (CO) groups as coordinated ligands and are used as catalysts in organic synthesis. Already metal carbonyl complexes are finding novel applications in research in the fields of cell biology, immunology, and pharmacology (Mosi et al. 2002). Their application in cancer therapy, drug receptor interaction, and malaria is also extremely promising. Bio-organometallic chemistry has now become an emerging discipline that may offer innovative solutions to biological problems. Progress in this field has been possible despite the fact that the majority of biologists and pharmacologists generally and incorrectly view transition metals ions as toxic 'heavy metals'. The

chemical development of anticancer drugs as well as immuno-suppressants based on ruthenium provides evidence of how metal-containing compounds can be used for the development of new pharmaceuticals (Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b).

From inorganic chemical studies conducted on these substances *in vitro*, it is known that certain ligands in a metal complex can promote, either sterically or electronically, the dissociation of CO. Furthermore, photodissociation and the consequent elimination of the CO group(s) following exposure to light has been reported in the case of specific metal carbonyls (Motterlini et al. 2002b). The discovery that carbonyl complexes posses such interesting and promising features *in vitro* prompted our laboratory to examine the ability of some of these compounds to promote a physiological response in biological systems. The molecules were initially tested to see if they released CO using a set up as in Figure 1.17 (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a). The release of CO was stimulated either by the exposure of the carbonyl solution to cold light in the case of iron pentacarbonyl and dimanganese decacarbonyl ($[Mn_2(CO)_{10}]$), or by direct addition to the myoglobin in the case of tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$). Due to the high affinity of myoglobin for CO any released would be captured by the myoglobin and the subsequent conversion of the deoxygenated myoglobin to carboxy myoglobin results in a change in the absorbance curve which gives a measure of the CO released (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002b).

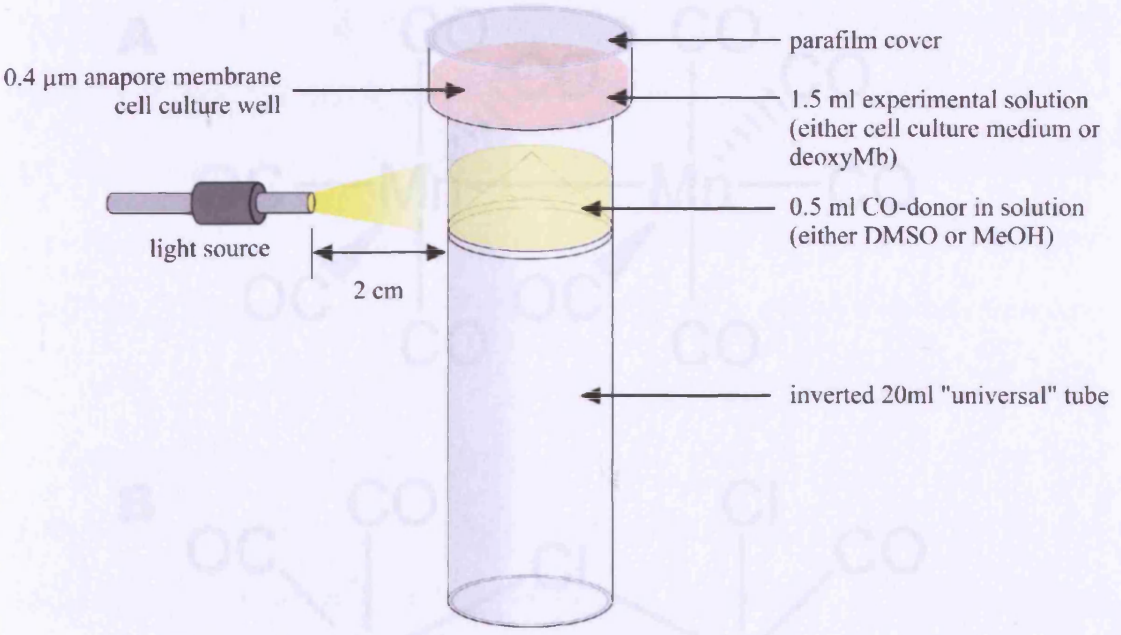


Figure 1.17:- Schematic diagram showing the CO release measurement setup for light stimulated compounds iron pentacarbonyl and dimanganese decacarbonyl.
CO released by the CO-RM would pass through a 0.4 µM Anapore™ membrane and into the myoglobin solution above. Adapted from Motterlini *et al*, Curr Pharm Des. 2003;9(30):2525-39.

At this point further investigations on iron pentacarbonyl were abandoned due to its deposition of a green brown precipitate. As there were no previous studies on the use of metal carbonyl complexes in biological systems the potential cytotoxic effects of each was evaluated. Neither $[Ru(CO)_3Cl_2]_2$ or $[Mn_2(CO)_{10})]$ caused any appreciable decrease in cell viability over time at concentrations below 400 µmol/L (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a). The structure of each is shown in Figure 1.18.

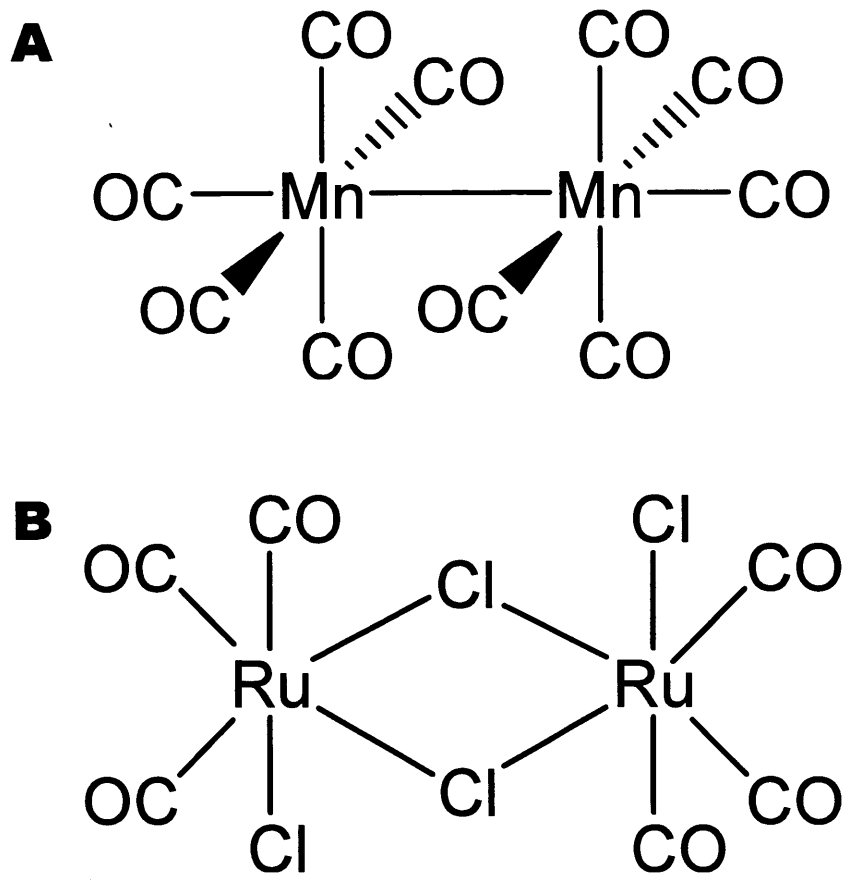


Figure 1.18:- Structure of A) dimanganese decacarbonyl ($[Mn_2(CO)_{10}]$) and B) tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$), CORM-1 and CORM-2 respectively.

The effect of $[Ru(CO)_3Cl_2]_2$ was then studied in an isolated aortic rings model (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a). The compound exhibited significant vasodilatory effects after the first addition (45% greater than control) and was shown to have a long term effect as phenylephrine induced contraction was not able to be restored even after extensive washings. This vasodilatory effect was significantly attenuated by the addition of myoglobin to the buffer (which as mentioned earlier avidly binds CO) and ODQ (a selective inhibitor of guanylate cyclase). Interestingly, ODQ only has an observed effect over the first two additions of $[Ru(CO)_3Cl_2]_2$ but the third

addition elicited a substantial vasodilatory effect despite the presence of ODQ. This data shows that CO released from this early identified CO-RM is at least in part cGMP dependent. The failure of ODQ to completely prevent vasodilation may result from CO being in sufficiently high levels to displace ODQ from guanylate cyclase or to circumvent this pathway altogether and exert relaxation via other independent cellular pathways (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002b).

Experiments using the isolated heart model and $[Mn_2(CO)_{10}]$ confirmed the vasoactive properties of this CO-RM further. This metal carbonyl markedly attenuated L-NAME mediated increase in coronary perfusion pressure; notably, this effect could only be achieved when $[Mn_2(CO)_{10}]$ was stimulated by light to release CO. This corroborates evidence showing that endogenously produced CO can profoundly modulate cardiac vessel function but was achieved using an exogenously applied CO-RM (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002b).

Further experiments were carried out investigating the antihypertensive actions of $[Ru(CO)_3Cl_2]_2$ in animals. As seen with previous experiments using hemin to upregulate HO-1, $[Ru(CO)_3Cl_2]_2$ was found to suppress the increase in mean arterial pressure elicited by the intravenous administration of L-NAME supporting further the potential of CO to be used *in vivo* (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002b).

These initial experiments opened the door to a host of exciting possibilities in the world of CO releasing molecules. In addition, with the success of exogenous CO gas delivery models in anti-inflammatory and anti-apoptotic the potential of a therapeutic application for CO-RMs was strongly emerging (Figure 1.19).

There was scope to engineer molecules that could release CO with distinct kinetics and have molecules that were highly specific and thus deliver CO directly to an area of insult. On the back of these first successful experiments our group went on to develop CO-RMs more compatible with the biological system.

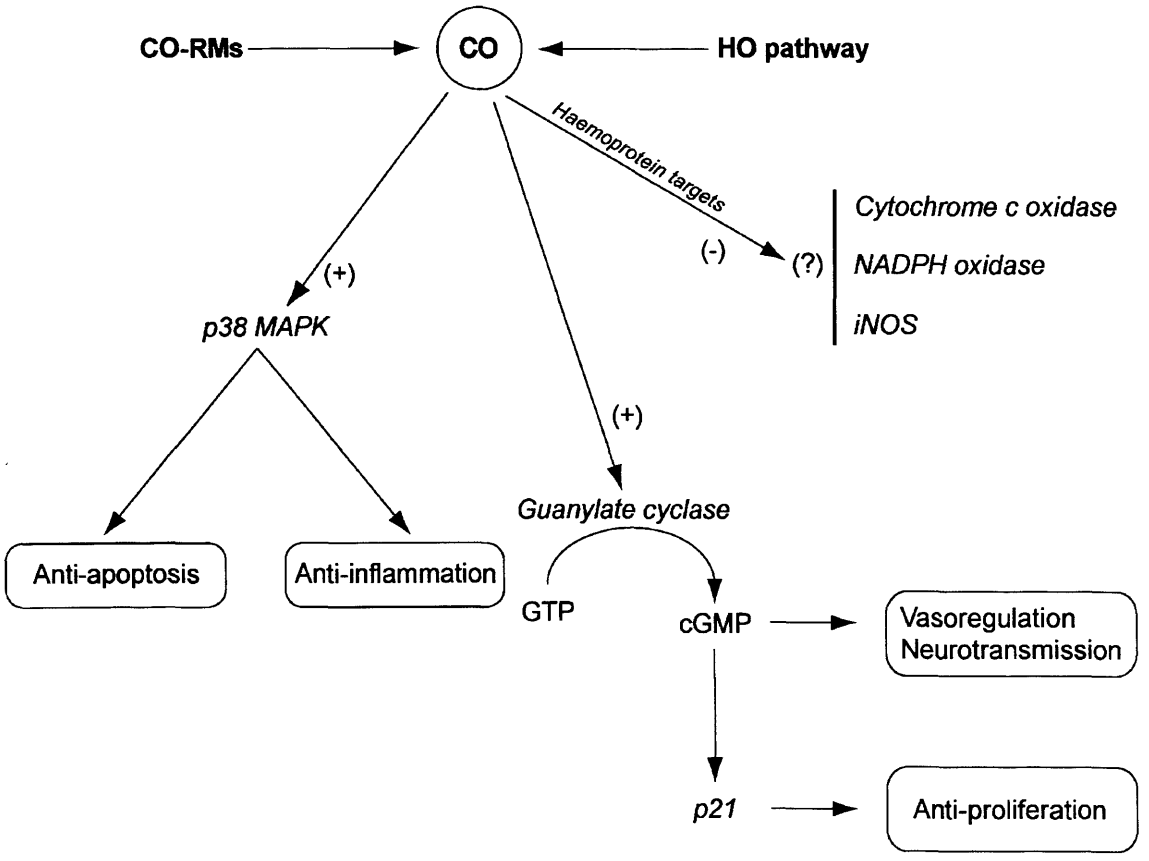


Figure 1.19:- Biological action of CO.
Endogenously derived CO has been shown to exert many effects. Most endogenously generated CO is derived from the HO pathway but could CO-RMs be used to achieve similar outcomes? Extract from Ryter *et al*, Mol Cell Biochem. 2002 May-Jun;234-235(1-2):249-63.

The original CO-RMs were only soluble in organic solvents and required physical (i.e. irradiating light) or chemical (i.e. steric ligands) intervention to favour CO dissociation from these complexes. Interestingly, the versatile

chemistry of transition metals allows them to be cleverly modified by coordinating biological ligands to the metal centre in order to render the molecule less toxic, more water soluble, and to modulate the release of CO. The first such modified molecule was Tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) which released CO and was water soluble (Figure 1.20) (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). The development of such a molecule was hugely important as it was biologically 'friendly' and could be used as a prototypic chemical in the development of pharmacologically active compounds capable of delivering CO for therapeutic purposes (Motterlini et al. 2003a; Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b).

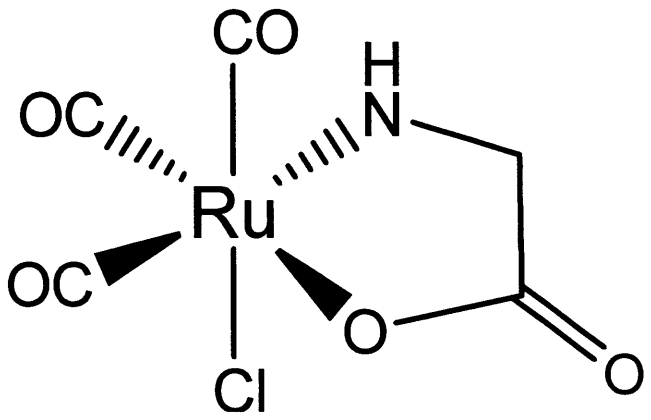


Figure 1.20:- Chemical structure of CORM-3, a water soluble CO-releasing molecule.

This first water soluble CO-RM has been extensively tested by our group and results obtained with this molecule in my studies will be examined later in this Thesis. Early investigation of CORM-3 involved testing the CO release using the myoglobin assay preparing the molecule in a number of solutions. The

liberation of CO from CORM-3 in biological systems occurs very rapidly (within 1-5 minutes (min)) (Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003a). Interestingly, it was found that CORM-3 remained stable in water (H_2O) left over night at room temperature; however, when prepared and left in Krebs buffer (pH = 7.4) in the same conditions it was unable to convert deoxy myoglobin to MbCO and was in effect inactive (iCORM-3) (Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b). Infrared spectroscopy suggests that iCORM-3 is a dicarbonyl species, consistent with the loss of one molecule of CO in its formation from CORM-3. This discovery presented us with a negative control for the compound which would aid in distinguishing between the effects of CO and the compound itself. CORM-3 was also shown not to cause any change in cell viability at concentrations between 10 and 50 μmol/L (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). This new water soluble CO-RM was shown to significantly attenuate the loss in viability of H9c2 cardiac cells exposed to hypoxia for 24 h and reoxygenation for 6 h (Clark et al. 2003a). This protective effect was not observed using iCORM-3 implicating CO as the mediator. The effect was totally abolished when 5-HD, an inhibitor of mitoK_{ATP} channels, was added. Opening of the channels has been shown to decrease intracellular calcium overloading and protect the heart against ischemia-reperfusion injury, and has been directly implicated in the mechanism of preconditioning associated with cardioprotection (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003a). Preservation of mitochondrial function during myocardial ischemia by the use of drugs such as nicorandil has also been attributed to activation of mitoK_{ATP} channels. The protective effect promoted by the opening of mitoK_{ATP} channels appears to be

linked to a delay in apoptosis as well as alteration in mitochondrial membrane potential. Notably, CORM-3 even preserved cell viability when added during the reoxygenation period. This protective effect was also demonstrated in cells exposed to paraquat, a superoxide anion generator used to promote oxidative stress (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003a).

These effects of CORM-3 were not limited to cell models. CORM-3 was used to protect hearts (male Lewis rats) from 30 min global ischemia and 60 min reperfusion in a Langendorff model. Predictably, iCORM-3 showed no such beneficial effects on hemodynamic, biochemical or histological parameters. In addition, the cardioprotective action of CORM-3 was significantly attenuated by the presence of 5-HD. As with the cell model this data demonstrated that CO was the likely mediator of action and that the activation of the mitoK_{ATP} channel was again involved in eliciting this response. CORM-3 was also used in a model of cardiac allograft rejection to verify its pharmacological action *in vivo*. The survival time of hearts transplanted into mice receiving CORM-3 was significantly prolonged with 40% of hearts still viable at 30 days. There was some protection by iCORM-3 but significantly less than that of CORM-3. This protection could indicate that *in vivo* the metal carbonyl is further metabolised to release the residual CO groups. This data demonstrated CORM-3 had the potential to attenuate organ rejection (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003a).

Since those initial experiments, CORM-3 has been studied in quite some detail. Its stability has been explored further and has been shown to remain inert in water and acidic pH for over 24 h, whilst, it has been demonstrated to release

CO in a number of physiological solutions and biological fluids such as PBS, Krebs buffer, cell culture medium and human blood plasma (Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b). It has also been found that CORM-3 is able to increase HO activity whilst iCORM-3 does not suggesting that some of the effects witnessed may be partially attributable to this phenomenon (Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003a).

The development of future CO-RMs which are more biologically suitable and able to release CO in a more targeted fashion continues. There are many aspects of CO-RMs that can be modified to suit the end user. For example, the use of various metal ions may elicit less of a toxic effect and aid in biological compatibility (i.e. iron). There is even the potential to synthesise non-metal containing CO-RMs as will be demonstrated later in this Thesis. Another key area of CO-RM development is the kinetics of CO release. By manipulating the ligands, the rate at which CO is released can be altered, allowing for slow delivery of CO over a period of time or a faster release where needed. CO-RMs that release CO very rapidly ("fast releasers") in biological systems would be ideal for therapeutic applications where CO acts as a prompt signaling mediator (i.e. neurotransmission, acute hypertension, angina, ischemia-reperfusion). However, the discovery of chemicals that release CO with a slow kinetic ("slow releasers") would implement the design of pharmaceuticals that could be more versatile in the treatment of certain chronic diseases (i.e. inflammatory states and chronic hypertension; rejection of transplanted organs) where the continuous and long-lasting effect of CO may be required. There are also various solubilities to consider. Currently water-soluble CO-RMs exist but the need for lipid soluble ones may also be considered. The most intriguing aspect

of CO-RM development is specific intra-cellular targeting. The molecules could be synthesised with receptor specific proteins or even some kind of antibody targeting which would allow for the delivery of CO to an exact target thus negating the need for a holistic CO release and any associated side effects of such an approach. The attempt to diversify the multiplicity of CO-RMs that possess a variety of chemical characteristics (i.e. water-soluble vs. lipid-soluble, slow vs. fast releasers) will help to elucidate the biological function of cellular targets that are responsive to CO and will facilitate in due course the design of versatile agents that could be used for the therapeutic delivery of CO in a safe, measurable and controllable fashion (Chatterjee 2004b).

1.12 Hypothesis and Objectives

1.12.1 Hypothesis

NO donors have had a fundamental role in the elucidation of the function of NO in the biological system. They have also offered a means of treating certain pathophysiological conditions associated with the role of NO in the body such as hypertension. In recent years, CO has been shown to exert many similar and coordinated responses to NO, thus, the development of compounds capable of releasing CO and exerting a physiological action is an attractive proposition. Metal carbonyls have been used for many years in pure chemical processes but the CO releasing potential of these compounds in a biological scenario was only investigated very recently (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a). Since initially identifying the first CO-RM, our group has continued developing further CO-RMs in conjunction with Professor Brian Mann at the University of Sheffield. Investigating and understanding the actions of CORM-3, the prototypic water soluble CO-RM, has resulted in the development of further CO-RMs with different chemical properties. Thus, will the biological activity of these CO-RMs allow the elucidation of the action of CO *in vitro* and *in vivo* and will they potentially have therapeutic value in pathophysiological conditions? Are the actions of CO-RMs elicited by the release of CO, the molecule itself, or by its effect on other biological pathways?

Overall Hypothesis

CO-RMs, through the action of CO, can mimick the action of endogenously genereated CO in a murine model of inflammation.

1.12.2 Objectives

This is a list of the objectives set out at the start of the PhD to assist in answering the hypothesis.

i) Test the prototypic water soluble CO-RM (CORM-3) to determine its toxicity in various cell lines, its effect on the HO system and its regulation of the inflammatory response inferred from TNF-α, nitrite and iNOS levels in macrophages.

ii) Investigate the ability of newly synthesised CO-RMs to release CO using the myoglobin assay to determine new CO-RMs with the potential to forward the study.

iii) Test compounds deemed to be worthy of further study from (ii) to determine their toxicity, effect on the HO system and regulation of the inflammatory response.

iv) Determine the CO releasing profile and bioactive properties of CORM-A1, a newly identified CO generator that does not contain a transition metal.

v) Demonstrate how structure can affect CO release, toxicity and nitrite using CORM-F compounds.

2 Materials and Methods

2.1 Reagents

Hemin (ferriprotoporphyrin IX chloride), tin protoporphyrin IX (SnPPIX) and biliverdin (biliverdin IX hydrochloride) were purchased from Porphyrin Products Inc (Logan, Utah, USA). Stock concentrations of SnPPIX (10 mM) and biliverdin (10 mM) were prepared in sodium hydroxide (NaOH) (0.1 M), kept on ice and used only on the day of treatment. Working stocks of hemin (2 mM) were prepared by solubilising in 1 ml 0.1M NaOH and making up to 5 ml using phosphate buffered saline (PBS) (pH = 7.4), kept on ice and used on the day of treatment. Sodium boranocarbonate ($Na_2[H_3BCO_2]$ or CORM-A1) was prepared as previously described by Alberto and coworkers (ALBERTO 2001). Tricarbonylchloro(glycinato)ruthenium (II) ($[Ru(CO)_3Cl(glycinate)]$ or CORM-3), CORM-319 ($[Fe(\eta\text{-}C_3H_5)(CO)_4][BF_4]$), and CORM-311 ($[Et_3NH][HFe_3(CO)11]$) were synthesised by Professor Brian Mann (University of Sheffield). Stock solutions of CORM-3 and CORM-A1 (10 mM) were freshly prepared before the experiments by dissolving the compounds in pure distilled water. CORM-319 (10 mM) was preprared in distilled water immediately prior to addition due to its inherent instability. CORM-311 was prepared as a 10 mM stock in reagent grade ethanol (Fisher). In our preliminary tests, we noticed that acidic pH significantly accelerate the spontaneous release of CO from CORM-A1. We therefore took advantage of this specific property of CORM-A1 and generated an inactive form (iCORM-A1) to be used as a negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N_2 through the solution for 10 min in order to remove the residual CO gas. The solution of iCORM-A1 was finally adjusted to pH = 7.4 and tested with the myoglobin assay

(Paragraph 2.4) prior to each experiment to verify its inability to liberate CO. Tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$ or CORM-2) was obtained from Sigma Aldrich. Dimethylsulphoxide (DMSO) was used to solubilise the compound to prepare a 10 mM stock. An inactive form of each compound was also used in this study. CORM-3 and CORM-319 were 'inactivated' by addition to cell culture medium and incubation at 37 °C in a 5% CO_2 humidified atmosphere for 18 h to liberate CO (iCORM-3 and iCORM-319). In the case of CORM-2 a related molecule ($Ru(DMSO)_4\ Cl_2$ or iCORM-2) where the carbonyl groups have been replaced with DMSO groups was utilised. Lipopolysachharide (LPS - *E.Coli* serotype 026:B6) was obtained from Sigma. Initially a 10 mg/ml stock was prepared, aliquoted and then stored at -80°C until required. Working stock solutions (0.1 mg/ml) were prepared by the addition of 10 µl of 10 mg/ml LPS stock to 990 µl sterile dH_2O (Sigma). Polyclonal antibodies against HO-1 were from Bioquote Ltd (York, UK) whereas polyclonal anti-iNOS antibodies were purchased from Santa Cruz Biotechnology Inc (Insight biotechnology, Wembley, Middlesex, UK). All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

2.2 Synthesis of Tricarbonylchloro(glycinato)ruthenium (II) (CORM-3)

Tricarbonylchloro(glycinato)ruthenium (II) ($[Ru(CO)_3Cl(glycinate)]$ or CORM-3) was synthesised starting from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (Sigma Aldrich). Briefly, $[Ru(CO)_3Cl_2]_2$ (0.129g) and glycine (0.039g) were placed under nitrogen in a round-bottomed flask. Methanol (75ml) and sodium ethoxide (0.034g) were added and the reaction was allowed to continue under stirring for 18 h at room

temperature. The solvent was then removed under pressure and the yellow residue redissolved in tetrahydrofuran (THF); this was filtered and excess 40 to 60 light petroleum added. The resulting yellow solution was evaporated down to give a pale yellow solid (0.142g, 96% yield). CORM-3 was stored in sealed vials at -20°C and used freshly on the day of experiment.

2.3 Synthesis of Sodium Boranocarbonate (CORM-A1)

H_3BCO was prepared from commercially available $H_3B{\cdot}THF$ solutions and reacted *in situ* with an alcoholic solution of sodium hydroxide to give CORM-A1 ($Na_2[H_3BCO_2]$). The key to the preparation is the control of equilibrium between H_3BCO and $H_3B{\cdot}THF$. THF is selectively condensed from the gas stream at -50°C, while H_3BCO (boiling point -64°C) passes on, carried by a stream of carbon monoxide. Subsequently, this gas mixture is directly bubbled though an ethanolic solution of NaOH at -78°C. Nucleophilic attack of $[OH]^-$ at the highly electrophilic carbon in H_3BCO leads to the formation of $Na_2[H_3BCO_2]$ in high yield. Carbon monoxide was slowly bubbled through 30 cm^3 of a 1 M $H_3B{\cdot}THF$ solution. The gas stream was passed over a reflux condenser at -50°C and then bubbled through a solution of 2.8 g NaOH in 200 cm^3 ethanol in a Schlenk tube at -78°C. After 2 h the Schlenk tube was disconnected and heated to reflux for about 1 h. $Na_2[H_3BCO_2]$ precipitates as a white solid in large amounts and was filtered and washed with cold ethanol and diethyl ester to obtain the pure product. CORM-A1 was stored in sealed vials at -20°C and used freshly on the day of experiment.

2.4 Detection of CO Release

The release of CO from metal carbonyl compounds was assessed spectrophotometrically by measuring the conversion of deoxy-myoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a; Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003a). The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient = 15.4 $mmol/L^{-1}$ cm^{-1}). Stock solutions of myoglobin (lyophilized horse heart) (Sigma) (66 µmol/L final concentration) were prepared fresh by dissolving the protein in PBS (0.01 M, pH = 7.4) (Sigma). Sodium dithionite (0.1%) (Sigma) was added to convert the myoglobin stock to deoxy-Mb prior to each experiment. A 1 ml quantity of this was measured to obtain a deoxy-Mb curve and then bubbled with CO to get a MbCO curve. CO-RMs (20, 40 or 60 µM) were added (5 µl) directly to the myoglobin in the cuvette (Sarstedt, Leicester, UK), mixed using a Gilson (1000 µl) and then overlaid with 500 µl mineral oil (Sigma) to prevent CO escaping or the myoglobin becoming oxygenated. Samples were immediately read on a spectrophotometer (Unicam Heλios α) and then read every 5 min for the first 30 min. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CO to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively (see Figure 2.1). Subsequent readings were taken every 30 min or when deemed necessary. Normally the readings were continued until the CO release reached a plateau for 2 consecutive readings. A step by step protocol for the myoglobin assay can be found in the appendix (paragraph 11.4).

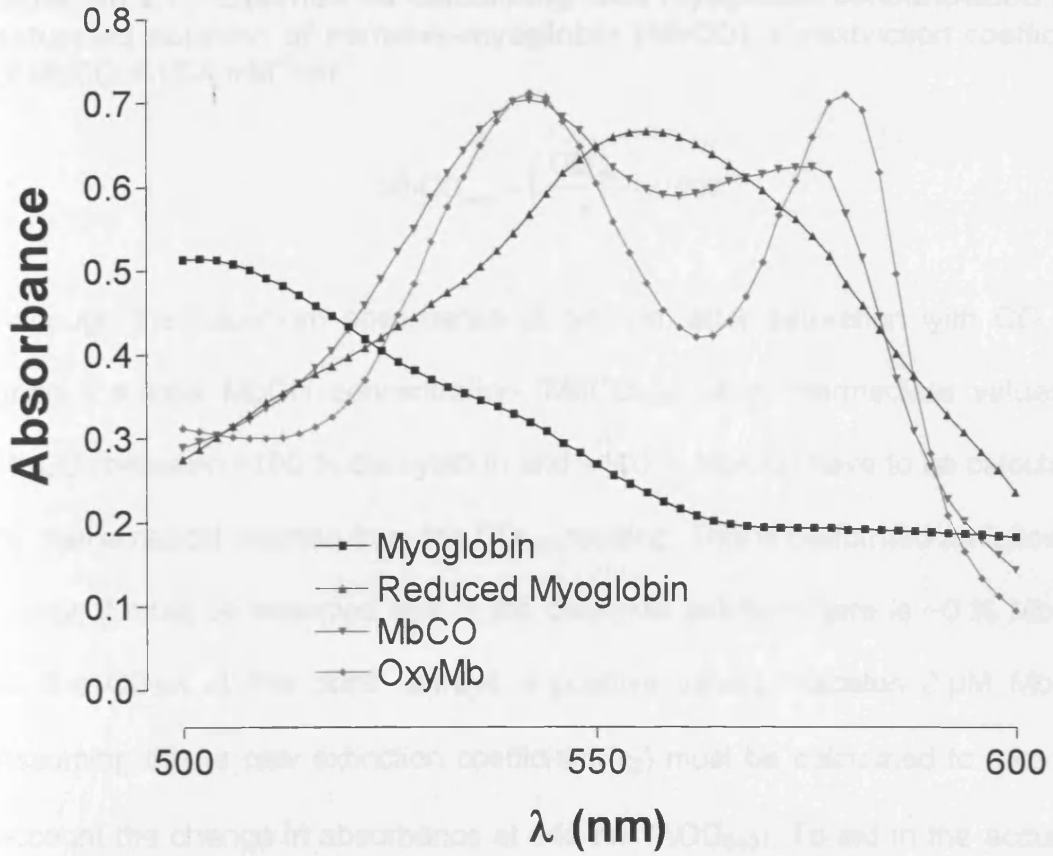


Figure 2.1:- The various spectra of myoglobin.
This figure shows the alternate spectra that are generated by reducing (sodium dithionite) or exposing myoglobin to carbon monoxide (MbCO) or 95% oxygen (OxyMb).

In order to calculate the total myoglobin in the solution, deoxyMb solution was bubbled for 5 min with 1 % CO gas until the absorbance at 540 nm reached a maximum. This OD value was taken as the maximum MbCO concentration and therefore the total myoglobin concentration of the mixture, although, in theory the solution of myoglobin was converted to ~100 % MbCO the measured amount was never as high as 66 μM (the initial calculated concentration of Mb) because the myoglobin used was 90% pure and thus contained impurities such as salts and denatured myoglobin in the original myoglobin solutions. Total myoglobin concentration was calculated using Equation 2.1.

Equation 2.1:- Equation for calculating total myoglobin concentration in a saturated solution of carmoxy-myoglobin (MbCO). ε = extinction coefficient of MbCO = 15.4 $mM^{-1}cm^{-1}$

$$MbCO_{max} = \left(\frac{OD_{540}}{\varepsilon}\right) x1000$$

Although the maximum absorbance at 540 nm after saturation with CO gas gives the total MbCO concentration ($MbCO_{max}$), any intermediate values of MbCO (between ~100 % deoxyMb in and ~100 % MbCO) have to be calculated by mathematical iteration from the OD_{540} reading. This is performed as follows: Firstly, it must be assumed that in the deoxyMb solution there is ~0 % MbCO, so the OD_{540} at this point (always a positive value) indicates 0 µM MbCO. Assuming this, a new extinction coefficient (ε_2) must be calculated to take into account the change in absorbance at 540 nm (ΔOD_{540}). To aid in the accuracy of this calculation, another wavelength is used as a constant reference point. Conveniently, the deoxyMb and MbCO spectra share four isosbestic (OD_{iso}) points between 500 and 600 nm (~510, ~550, ~570 and ~585 nm). The value at 510 nm (OD_{ISO510}) was taken for these set of experiments. Thus, the new extinction coefficient was calculated:

Equation 2.2:- Equation needed to calculate an unknown MbCO extinction coefficient.
Taking into account the change in absorbance at the isosbestic point (ΔOd_{iso510}) and the change in absorbance at 540 nm (ΔOD_{540}) a new extinction coefficient (ε_2) can be calculated.

$$\varepsilon_2 = \left(\frac{\Delta OD_{540} - \Delta OD_{iso510} \times 1000}{MbCO_{max}}\right)$$

From these calculations, the concentration of MbCO in a solution of myoglobin can be calculated if three things are known about the solution: i) the change in OD_{540} between deoxy- and saturated MbCO (by saturating the Mb solution with CO gas); ii) the change in the OD_{iso} in the sample from original deoxyMb solution and; iii) the OD_{540} and OD_{iso} of the sample solution. The following equation is therefore used to calculate the [MbCO] of the unknown sample:

Equation 2.3:- Calculation of unknown MbCO concentrations.
Where ΔOD_{540} is the change in absorbance at 540 nm, ΔOD_{iso} is the change in absorbance at the isosbestic point, $MbCO_{max}$ is the MbCO concentration calculated after saturation of the Mb with CO gas and ε_2 is the calculated absorption coefficient.

$$MbCO = \left(\frac{\Delta OD_{540} - \Delta OD_{iso510}}{\varepsilon_2} \right) \times 1000$$

After all the time points have been calculated, the data can be converted from a concentration (μM) to absolute MbCO values (nmoles) by multiplication of the μM value by the volume of myoglobin in the solution (in l). From a graph of MbCO (nmoles) vs. time (min), the incubation time required for any MbCO concentration can be calculated; therefore cells can be exposed to a known concentration of CO using this system.

2.5 Cell Culture

Murine RAW264.7 monocyte macrophages were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and cultured in complete medium consisting of: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml

penicillin and 0.1 mg/ml streptomycin. Initially the existing medium in each flask was removed and discarded. Cells were then washed with 5 ml warm (37 °C) 10x PBS (Gibco, Paisley, UK) which was replaced with 8 ml warm (37 °C) DMEM and the cells scraped using a rubber plastic Thomas policeman (Thomas Scientific, Swedesboro, NJ) to remove them from the flask surface. The cells were then pipetted (2 ml) into 4 new T-75 flasks (Sarstedt, Leicester, UK) each containing 8 ml DMEM and returned to the incubator (LEEC).

Bovine aortic endothelial cells were purchased from Coriell Cell Repositories (Camden, NJ, USA) and cultured in complete medium consisting of: Iscoves modified Dulbecco's medium which was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were sub-cultured approximately every 3 days to maintain the cell line for subsequent experiments. Confluent cells (>80% coverage of 75 cm^2 flask) were firstly washed with 5 ml of warm (37 °C) PBS to facilitate the action of trypsin (0.25% trypsin-EDTA solution, Sigma). Once the PBS was removed, trypsin (also 37 °C) was added to remove cells from the surface of the flask. Cells were then briefly incubated at 37 °C for approximately 3 min to allow the trypsin to act. The trypsin (now including the cells) was removed and pipetted into 8 ml of fresh medium to neutralise its action and prevent over exposure of the cells to this agent which could be detrimental. Cells were then centrifuged (1500 x *g*) for 5 min in a bench top centrifuge (SANYO, Harrier 15/80). The supernatant was removed and fresh medium was added to re-suspend the pellet (a 1:3 split would require 6 ml to re-suspend the pellet). Then, 2 ml of the re-suspended cells were pipetted into pre-prepared T-75 flasks containing 8 ml fresh medium and incubated. Over an extended period of slow growth where cells failed to

reach confluence within 3-4 days, the medium was replaced with fresh medium. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

Girardi (human heart) cells and wild type smooth muscle cells (bovine) were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and cultured in complete medium consisting of: Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 10% fetal bovine serum, 3.5 mM L-glutamine, 1% non-essential amino acids (NEAA), 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were sub-cultured approximately every 3 days to maintain the cell line for subsequent experiments. Confluent cells (>80% coverage of 75 cm^2 flask) were firstly washed with 5 ml of warm (37 °C) PBS to facilitate the action of trypsin (0.25% trypsin-EDTA solution, Sigma). Once the PBS was removed, trypsin (also 37 °C) was added to remove cells from the surface of the flask. Cells were then briefly incubated at 37 °C for approximately 3 min to allow the trypsin to act. The trypsin (now including the cells) was removed and pipetted into 8 ml of fresh medium to neutralise its action and prevent over exposure of the cells to this agent which could be detrimental. Cells were then centrifuged (1500 x *g*) for 5 min in a bench top centrifuge (SANYO, Harrier 15/80). The supernatant was removed and fresh medium was added to re-suspend the pellet (a 1:3 split would require 6 ml to re-suspend the pellet). Then, 2 ml of the re-suspended cells were pipetted into pre-prepared T-75 flasks containing 8 ml fresh medium and incubated. Over an extended period of slow growth where cells failed to reach confluence within 3-4 days, the medium was replaced with fresh medium.

Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

All cell culture work was carried out using an ESB JBIO CL II category II hood.

2.6 Subculture of Cells into 24 Wells

Cells were prepared as per paragraph 2.5 up until the pellet was to be re-solubilised (BAEC, Girardi) or the cells had been scraped (RAW264.7). At this stage the pellet was re-solubilised using 2 ml of complete medium whilst the macrophages were left as they were. From either cell suspension, 2 ml was then added to a pre-prepared Falcon tube (50 ml, NUNC) containing 49 ml of complete medium. From this, 2 ml was added to each well of a 24 well plate, with any remaining cell / medium suspension discarded. The plate was then incubated at 37 °C until confluent.

2.7 Subculture of Cells into 6 Wells

Cells were prepared as per paragraph 2.5 up until the pellet was to be re-solubilised (BAEC, Girardi) or the cells had been scraped (RAW264.7). At this stage the pellet was re-solubilised using 3 ml of complete medium whilst the macrophages were left as they were. From either cell suspension, 3 ml was then added to a pre-prepared Falcon tube (50 ml, NUNC) containing 47 ml of complete medium. From this, 2 ml was added to each well of a 6 well plate, with any remaining cell / medium suspension discarded. The plate was then incubated at 37 °C until confluent.

2.8 Preparation of Liver Cytosol and Liver Microsomes

Liver cytosol was obtained from male Sprague Dawley rats (250-300 g). Following sacrifice by anaesthetization with phenobarbital Lethobarb® sodium BP (500 µl, i.p.) and cervical dislocation, a 50 ml syringe was used to perfuse cold (4°C) buffer (1.15% (w/v) KCl) through the liver lobes to remove any residual blood, while clotted tissue was discarded. The organ was weighed, cut finely with scissors and homogenised in 2-3 volumes of homogenising buffer (20 mM Tris-HCl, pH = 7.4, containing 1.15% (w/v) KCl), and then shared out equally into a set of polyallomer centrifuge tubes (Beckman Coulter Ltd., High Wycombe, UK). After centrifuging (5,000 x *g*) for 20 min at 4°C in an ultracentrifuge (Beckman LE-80 ultracentrifuge) the supernatant was retained and then re-centrifuged (105,000 x *g*) for 90 min at 4°C. Following each centrifugation, the turbid lipid layer was removed with a Pasteur pipette. The supernatant was collected, and protein and haem oxygenase activity were measured as described in paragraph 2.13 and 2.10, respectively, then aliquoted and stored at -80°C.

The preparation of liver microsomes is analogous to that for liver cytosol with the following variations. The rat was pre-treated with hemin (50 mg/kg, i.p.) 24 h prior to harvest to induce HO. The lobes were perfused with cold (4°C) saline (0.9% (w/v) NaCl) and homogenised in 5 volumes of sucrose solution (0.25 M sucrose, 0.05 M Tris-HCl, pH = 7.4) then centrifuged. After the last centrifugation step, the resulting microsomal pellet was resuspended gently in 1 ml of phosphate buffer (pH = 7.4). Protein and haem oxygenase activity were determined according to the protocols described in paragraph 2.13 and 2.10, respectively, followed by storage at -80°C.

2.9 Collection of Samples

Before each experiment, cells were washed with 10 ml Dulbecco's PBS at 37 °C to remove cell debris, detached cells or unwanted chemical factors in the culture. After each treatment, confluent cells were washed with 5 ml cold (4°C) PBS (10x), and the buffer removed. Cold (4°C) PBS (5 ml) (1x) was then added and cells were gently scraped using a rubber plastic policeman. The cell suspension was transferred to a 14 ml plastic tube (Sarstedt, Leicester, UK) and a further 5 ml PBS was added to the flask to collect the remaining cells. Samples were then centrifuged (1800 x *g*) (SANYO, Harrier 15/80) for 10 min (4 °C). The supernatant was removed and cells were re-suspended with either: 1) 100mM PBS - 2mM $MgCl_2$ buffer (550 µl) for the HO activity assay or 2) 1% Triton PBS (300 µl) for the Western blot. Samples were stored at –80°C until the assays were performed.

2.10 Determination of Haem Oxygenase Activity

Stock solutions of the assay reagents, i.e. glucose-6-phoshate (G6P) (20 mM), glucose-6-phoshate dehydrogenase (G6PDH) (50 U/ml) and NADPH (40 mM), were prepared with phosphate buffer (0.01 M, pH = 7.4) and stored at -80°C until required. The NADPH was wrapped in foil as it is light sensitive. Hemin (2 mM) was prepared freshly for each assay using 0.5% (v/v) NaOH (2 M) and phosphate buffer (pH = 7.4). Rat liver cytosol and liver microsomes were prepared according to paragraph 2.8.

The samples, collected according to paragraph 2.9, were disrupted by three rounds of freeze-thawing (-80 °C to 37 °C) then placed on ice. A 100 µl aliquot was retained for protein determination according to paragraph 2.13. To a set of

glass tubes, which were maintained on ice, the cell suspension (400 µl) was added to the reaction mixture (final volume, 900 µl) containing: phosphate buffer (pH = 7.4), 20 µM hemin, 2 mM G6P, 0.5 U/ml G6PDH, 3 mg of rat liver cytosol, as a source of biliverdin reductase (BVR), and 0.8 mM NADPH. The negative and positive controls (rat liver microsomes) contained proportionate volumes of phosphate buffer (pH = 7.4) instead of the cell suspension. The tubes were vortexed well and incubated in the dark for 1 h at 37 °C. The activity of the cytosolic and microsomal fractions was verified by replacing hemin with biliverdin (1 mM), and the samples incubated in the dark for 30 min at 37 °C.

The reaction was terminated by the addition of chloroform (1 ml), then mixed thoroughly by vortexing, followed by centrifuging (1800 x *g*) 2 times for 5 min at room temperature using the bench-top centrifuge (SANYO, Harrier 15/80) until three distinct layers were formed. The lower organic (clear) layer, which contains the chloroform and bilirubin, was placed in a quartz cuvette and the difference in absorption between 464 nm and 530 nm (extinction coefficient (ε) for bilirubin in chloroform, 40 $mM^{-1}cm^{-1}$) was measured with the UVikon 810P spectrophotometer (Kontron Instruments) against a blank of chloroform. Haem oxygenase activity was expressed as picomoles of bilirubin formed per mg cell protein per h using Equation 2.4.

Equation 2.4:- Calculation of haem oxygenase activity.

$$\frac{\text{pmoles bilirubin}}{\text{mg protein in 60 min}} = \left(\frac{\Delta OD\ (OD_{464} - OD_{530})}{40} \Big/ \text{mg protein} \right) \times 10^6$$

2.11 Glutathione Measurement

To measure the glutathione concentration of treated cells and hence infer the redox levels, a glutathione assay was carried out as previously described by our group (FORESTI 1997). The medium of treated 6 well plates was discarded and cells were washed for 5 min with 2 ml of warm (37 °C) sterile 10X PBS. The PBS was removed and replaced with 600 µl of a 2 % (w/v) 5-sulfosalicylic acid solution, for cell lysis and deproteinisation, which was prepared in distilled water. Cells were then scraped and the cell suspension centrifuged (Beckman Avanti™ 30 centrifuge) at 10,000 g for 5 min at 25°C. The supernatant (500 µl) of each sample was removed and added to a cuvette containing 500 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) buffer and allowed to react for 5 min. DTNB buffer was prepared fresh each time and consisted of 0.3M sodium phosphate buffer ($NaHPO_4$ $7H_2O$), ethylenediamine tetraacetic acid (EDTA) 10 mM and DTNB 0.2 mM prepared in distilled water (pH = 7.5). Samples were then read on a UVikon 810P spectrophotometer at 412 nm blanking against a cuvette containing 500 µl 5-sulfosalicylic acid and 500 µl DTNB. Positive and negative controls consisted of cells treated for 24 h with 100 mM N-acetyl-cysteine (NAC), a precursor of glutathione, and 100 mM DL-butathione-[S,R] sulfoxide, a selective inhibitor of glutathione biosynthesis, respectively. The concentration of each sample was determined as in Equation 2.5 where the extinction coefficient (ε) was 14.3 mM^{-1} cm^{-1}.

Equation 2.5:- Calculation of glutathione concentration

$$\text{Concentration} = \frac{\text{Absorbance}}{\varepsilon}$$

2.12 Assay for Nitrite Levels

Nitrite levels were determined using the Griess assay protocol, a colorimetric assay. The medium from treated cells cultured in 24 well plates was removed and centrifuged (Beckman Avanti™ 30 centrifuge) at 10,000 g for 5 min at 25°C to remove any cells before it was plated out on to a 96 well plate in triplicate (50 µl per well). In addition 50 µl of standards (prepared using sodium nitrite - $NaNO_2$) (0 µM to 300 µM) were added to the plate in triplicate (Figure 2.2).

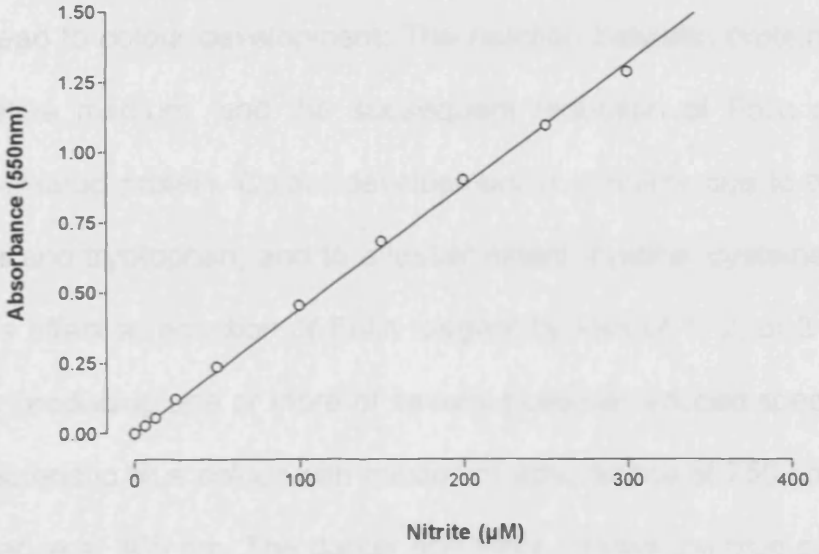


Figure 2.2:- Sample standard curve for nitrite assay

Next, 50 µl of Griess reagent (125 mg of sulphanilamide and 12.5 mg of N-(1-naphtyl)ethylenediamine (dihydrochloride) (Sigma) dissolved in 5 ml of dH_2O and 0.735 ml of orthophosphoric acid (H_3PO_4) (85%), the total volume was then brought up to 25 ml using dH_2O) was added to each well to begin the reaction. The plate was then shaken for 10 min to allow the reagents to mix and then the absorbance read at 550 nm on a plate reader (Molecular Devices VERSAmax

tunable microplate reader). Nitrite levels of each sample were determined using the standard curve.

2.13 Protein Determination

The level of protein in each sample was determined colourimetrically with the aid of the *DC* Protein Assay (Bio-Rad Laboratories Ltd). The assay is similar to the Lowry assay and is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to colour development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm. The darker and more intense the blue colour the higher the protein concentration. Samples were prepared for their respective assays i.e. freeze / thawing. Initially the working reagent was prepared which is either entirely reagent A if the samples contain no detergent (i.e. haem oxygenase assay) or supplemented with reagent S (4% v/v) if the samples do contain detergent (i.e. western blot analysis). To determine protein levels, 100 µl of each sample and 500 µl of working reagent were pipetted into clean polypropylene test tubes and the contents briefly vortexed. A blank was also run where 100 µl of the buffer used to prepare the sample was used in place of the sample. Next, 4 ml of reagent B was dispensed into each tube and vortexed

immediately. The samples were then left for 20 min to allow for colour development after which the absorbance's were read at 750 nm on a spectrophotometer (UVikon® 810 P). When running protein assays a number of standards (bovine serum albumin) were also run to prepare a standard curve (Figure 2.3). The standards, of which there were 5-6 dilutions containing between 0.2 µg/ml and 1.5 µg/ml protein, were treated in the same way as the samples.

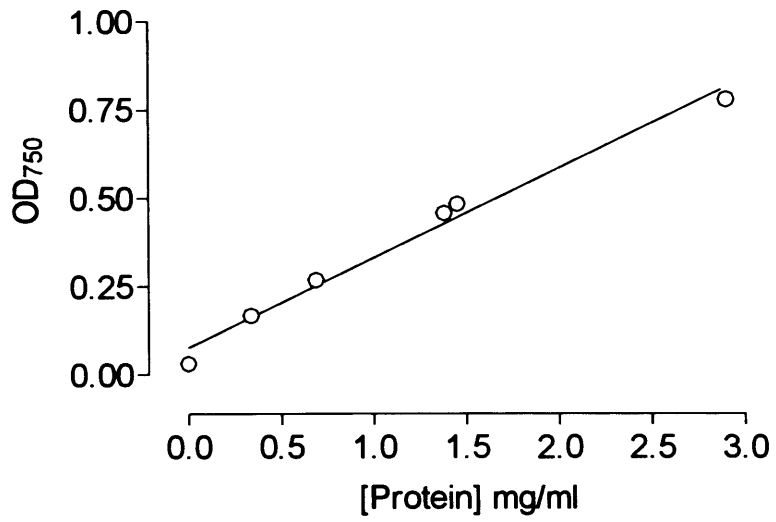


Figure 2.3:- Sample standard curve for protein assay

2.14 Cell Viability Determination

To determine cell viability three independent techniques were carried out each quantifying a particular aspect of cell health. The combination of all three gives a good measure of the overall viability of cells subsequent to treatment.

2.14.1 Alamar Blue Assay

Cell metabolism was determined using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK). The medium of treated cells was removed from all wells on the plate(s) and replaced by 1 ml of a 10% Alamar Blue : 90% complete cell culture medium mixture. The plate(s) was then left to incubate for 4 h after which 200 µl from each well was loaded on to a 96 well plate to be read on a plate reader (Molecular Devices) at 570 nm (subtracting any background absorbance present at 600 nm). The assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised form (blue) to a reduced form (red). It has been shown that ionophores, a molecule which carries or transports charged compounds or ions across cell membranes, block the reduction of Alamar Blue preventing its reduction in cell free extracts. It will, however, accept electrons from the electron transport chain. The Eh (oxidation-reduction potential) of Alamar Blue is similar to that of the cytochromes. The redox dye in Alamar Blue accepts electrons from the electron transport chain in a similar fashion to the terminal reduction of oxygen. The reduced form of Alamar Blue is secreted out of the cell and back into the media.

The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control.

2.14.2 Trypan Blue Assay

The potential deterioration of the plasma membrane of cells exposed to various treatments was examined using trypan blue (Sigma, UK). The dye permeates cells in which the cell membrane has been breached indicating a loss of viability. The medium of treated cells was removed from all wells on the 6 well plate(s) and discarded. It was replace with 0.5 ml of a 1:1 solution of trypan blue and warm (37 °C) 1X PBS. The 1X PBS was prepared as a 1 in 10 dilution of 10X PBS in sterile distilled water. The cells were then incubated at 37 °C and 5% CO_2 for 15 min after which the trypan blue was removed and replaced with 1 ml of warm (37 °C) 1X PBS. Cells were then viewed under a Olympus Tokyo CK microscope (20 X Magnification) and the ratio of cells stained blue (in which the dye has been taken up) to cells which remain unstained (and thus have an intact cell membrane) used to determine the percentage that are not viable.

2.14.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH based cytotoxicity detection kit (Roche Diagnostics, UK) gives a method for the colourimetric assay for the quantification of cell death and cell lysis based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The

assay was carried out according to the manufacturer's instructions. Upon completion of treatment the medium of each group was spun (Beckman Avanti™ 30 centrifuge) at 500 g for 5 min at 25°C to remove any cellular debris. Cell free culture supernatant (100 µl) was transferred to a clear flat bottom 96 well plate (NUNC, UK) to which 100 µl of reaction mixture was added. The reaction mixture consisted of a lysophilised catalyst which was reconstituted in 1 ml of distilled water and an INT dye. The ratio of catalyst to dye was 1:46. The plate was then incubated at 37 °C and 5% CO_2 for 15 min and protected from light. Samples were read on a plate reader (Molecular Devices VERSAmax tunable microplate reader) at 490 nm with a reference wavelength of 690 nm and blanked against cell culture medium. The percentage cytotoxicity was determined as in Equation 2.6. Samples were run in triplicate and a 1 % triton / medium mixture was added to some wells as a positive control, since triton degrades cell membranes and hence releases maximal LDH activity.

Equation 2.6:- Calculation of LDH activity associated cytotoxicity

$$\text{Cytotoxicity (\%)} = \frac{\text{Exp. Value} - \text{Low Control}}{\text{High Control} - \text{Low}} \times 100$$

2.15 Molecular Biology Procedures

2.15.1 Western Blot Protein Analysis

Total protein of each sample was calculated as per paragraph 2.13. A general sequence of events for the Western Blot can be seen in Figure 2.4.

Figure 2.4:- The sequence of antibody-substrate incubations for Western blot.
1) the nitrocellulose membrane will contain a number of antigens; 2) a specific polyclonal (raised in rabbit) antibody to the required antigen is bound; 3) anti-rabbit IgG with a biotin tail is then bound to the 1° antibody; 4) Extravidin alkaline phosphatase specifically binds to the biotin and, finally; 5) addition of the substrate gives a red precipitate indicating the presence of the antigen. (Figure designed by Dr James Clark).

An appropriate volume of each unknown sample was combined with Laemmli (loading) buffer (Bio-Rad Laboratories Ltd.), containing 5% (v/v) β-mercaptoethanol (total volume 30 µl), so that the final concentration loaded onto each lane was equal to 30-40 µg protein. The HO-1 positive control (Bioquote Ltd., York, UK) was diluted to 1 µg/ml in loading buffer. Samples were pulsed for 20-30 seconds (sec) on the bench-top microcentrifuge (SANYO MSE Micro Centaur) followed by denaturing of the proteins for 10 min at 90°C on a heating block (Techne DB2A, Techne GmBH, Germany). The molecular weight marker (MWM) (Invitrogen LifeTechnologies Ltd.) was not heated but placed on ice until required. After heating, the samples were re-pulsed and 30 µl of samples, 15 µl of MWM and 30 µl positive control were loaded carefully using gel loading tips (Fischer, UK) into the wells of a 12% tris-glycine Ready gel (Bio-Rad Laboratories Ltd.). Electrophoresis was carried out at room temperature in a tank containing running buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS) (Fischer, UK) using the Mini-PROTEAN® II system and Power-Pac 300 power supply (Bio-Rad Laboratories Ltd.) at a constant voltage of 120 V until the loading buffer had migrated to the base of the gel.

While the gel was running appropriately sized sheets of nitrocellulose membrane 45 µm pore size (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and 3MM Whatman™ blotting paper were cut and presoaked in dH_2O and transfer buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS, 20% (v/v) methanol) respectively for 10 min. After electrophoresis was completed, a 'gel sandwich' (see Figure 2.5 for assembly) was prepared upon the clear (positive electrode) side of a gel cassette within a shallow vessel containing some transfer buffer. After removal of any air bubbles, the cassette

was placed into the electrode module with the black (negative electrode) side facing the black (cathode) panel of the module. This orientation ensures the proteins migrate from the gel onto the nitrocellulose membrane. Extra transfer buffer was added and overnight transfer was carried out at 4 °C in a cold room with a constant voltage of 30 V using a 1000/500 transfer unit and Mini Trans-Blot® electrophoretic transfer cell, both from Bio-Rad Laboratories Ltd.

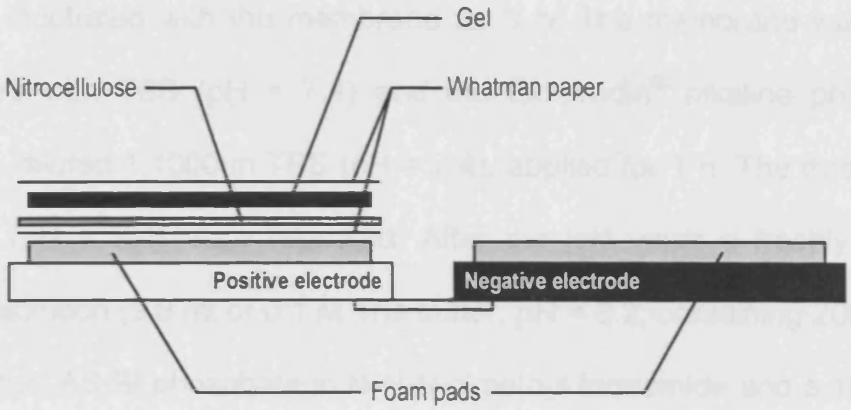


Figure 2.5:- Arrangement of gel sandwich assembly.

All subsequent procedures were performed at room temperature on an orbital shaker (Denley Instruments Ltd., Billingshurst, Sussex, UK) at low speed. Non-specific binding (NSB) of the antibodies was prevented by incubating the membrane in blocking solution (5% (w/v) non-milk dried fat in PBS (0.01 M phosphate buffer, pH = 7.4, containing 0.2% Tween-20), for 2 h at room temperature. This was followed by a single 5 min wash with 10 ml of PBS (pH = 7.4). All subsequent washes were for 5 min with 10 ml of the named solution. The membrane was incubated for 2 h with the anti-HO-1 primary antibody (Bioquote Ltd., York, UK) diluted 1:1000 in tris buffered saline (TBS) (0.05 M Tris-HCl, 0.0037 M KCl, 0.7137 M NaOH, pH = 7.4). When iNOS protein was

analysed the antibodies, which were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc, Insight biotechnology, Wembley, Middlesex, UK) were diluted (1:1000) in TBS. Next, the membrane was washed three times, once with PBS-T (PBS 0.01 M, pH = 7.4, containing 0.05% (v/v) Tween 20), then twice with TBS (pH = 7.4).

Proteins were visualised using an ExtrAvidin® alkaline phosphatase staining kit (Sigma). The biotinylated anti-rabbit IgG antibody, diluted 1:1000 in TBS (pH = 7.4), was incubated with the membrane for 1 h. The membrane was washed three times with TBS (pH = 7.4) and the ExtrAvidin® alkaline phosphatase conjugate, diluted 1:1000 in TBS (pH = 7.4), applied for 1 h. The three washes with TBS (pH = 7.4) were repeated. After the last wash a freshly prepared substrate solution (9.8 ml of 0.1 M Tris buffer, pH = 8.2, containing 200 µl of 1% (w/v) Napthol-AS-BI phosphate in N-N-N-dimethyl formamide and a 10 mg Fast Red DT salt tablet) was incubated with the membrane until red/pink bands, which indicates the location of the antigen-antibody complexes of the protein on the nitrocellulose membrane, was observed. After maximal colour development was attained, any excess substrate solution was washed off with distilled water (dH_2O) and the membrane stored in foil, until scanned into a PC (Epsom – Epsom Perfection 1650).

2.15.2 Enzyme-Linked Immunosorbent Assay for Cytokines

The quantification of IL-10, IL-6 and TNF-α in cell culture supernatants was determined using an enzyme-linked immunosorbent assay kit and carried out according to the manufacturers instructions (R&D systems, UK). The basic principle of an ELISA is shown in Figure 2.6.

Figure 2.6:- The principle of the ELISA system.
1) Wells are coated with a polyclonal antibody specific for mouse TNF-α; 2) the TNF-α present in the samples or standards binds to the primary antibody forming a stable complex; 3) a polyclonal antibody against mouse TNF-α conjugated to horseradish peroxidase is added which binds the TNF-α bound to the primary antibody ; 4) the substrate (3, 3', 5, 5' - tetramethylbenzidine/hydrogen peroxide), reacts with the antibody-bound peroxidase-labelled TNF-α causing a change in colour in the well. (Based on a figure designed by Dr James Clark).

Treated cell culture supernatant was removed and spun at 500 g for 5 min at 25°C (Beckman Avanti™ 30 centrifuge) to remove any cellular debris. Assay dilutent (50 µl) was added to each well on the plate, pre-coated with the corresponding antibody relating to the cytokine of interest, prior to the addition of the standards, control and samples (50 µl). The samples were gently mixed by tapping for 1 min, covered with an adhesive plastic cover strip and incubated for 2 h at room temperature. Each well was then aspirated and washed with 400 µl of wash buffer in a process that was repeated a total of 5 times. After the last wash, any remaining wash buffer was aspirated and the plate blotted against

clean paper towels. Following the washing, an enzyme-linked polyclonal antibody specific to the cytokine of interest was added (100 µl) to each well. The plate was covered with an adhesive strip and incubated at room temperature for 2 h. As before the plate was aspirated and washed five times. Substrate solution (100 µl) was then added to each well and the plate incubated at room temperature for 30 min in the dark. To halt the reaction, 100 µl of stop solution was added to each well and the absorbance read at 450 nm (correction wavelength of 570 nm) on a Molecular Devices VERSAmax tunable microplate reader. Cytokine concentration was determined by plotting the unknown samples on a standard curve of known cytokine concentrations (Figure 2.7).

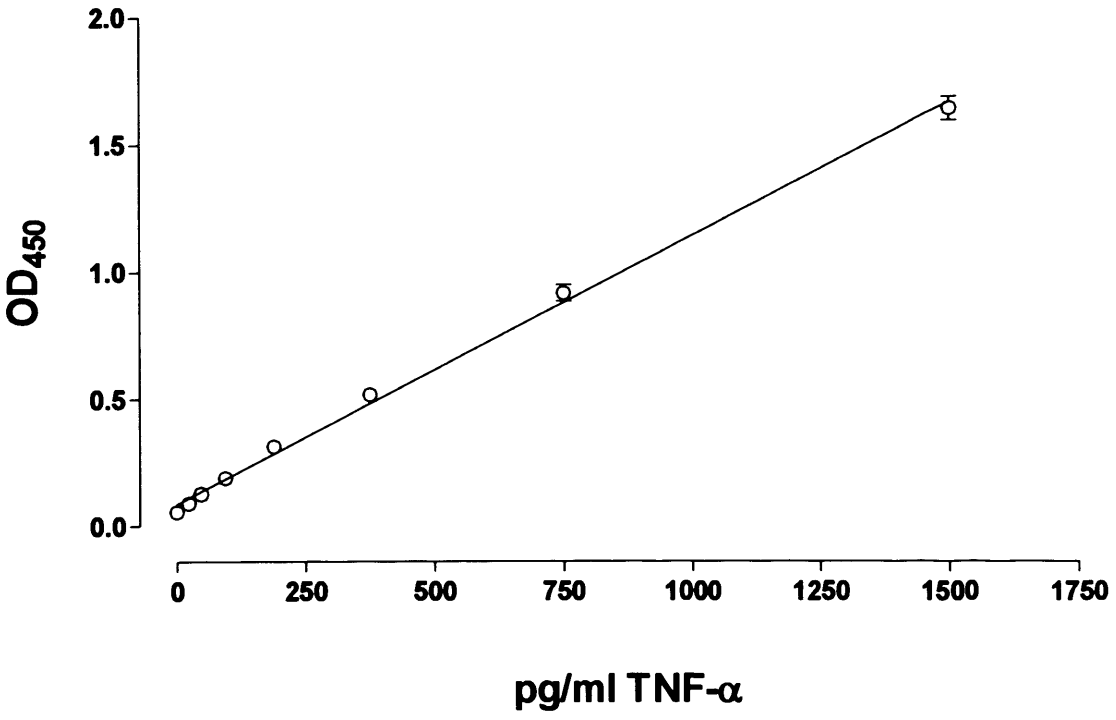


Figure 2.7:- Standard curve from TNF-α ELISA

2.16 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with the Bonferroni test where more than two treatments were applied. Differences were considered to be significant at $P < 0.05$. The measurement of only a single variable during different treatments meant the use of a non-parametric statistical test such as one-way ANOVA was appropriate. To compare the statistical significance of one treatment to another a Bonferroni post test was used subsequent to the ANOVA. All statistics were carried out using the Graphpad computer software PRISM version 4. Prism evaluates the data to determine if it is normally distributed prior to running a statistical analysis. Data which is unsuitable for the specific statistical test to be used is not analysed.

3 Chapter 3: Bioactivity of CORM-3

3.1 Introduction

Ever since NO was characterised as the EDRF, it has been studied in minute detail (Ignarro, Buga, Wood, Byrns, & Chaudhuri 1987; Grisham, Jourdheuil, & Wink 1999). The studies into the biological function of NO were made significantly easier with the development of NO donors, compounds capable of releasing NO in a biological environment (Willmot et al. 2005; Al Sa'doni & Ferro 2005; Low 2005). The biological activity of NO donors is exemplified by the use in the clinical setting of organic nitrites in the treatment of pathophysiological conditions such as systemic and pulmonary hypertension, angina and vascular dysfunction (Napoli & Ignarro 2003).

Not long after NO was established as an important signaling mediator, Marks *et al* suggested that CO may play a similar role (Marks, Brien, Nakatsu, & McLaughlin 1991). After all, both are diatomic gases of a similar molecular weight and both the HO pathways (CO generation) and NOS pathways (NO formation) seemed to be not only alike in components (inducible and constitutive isozymes) but were also inextricably linked and shown to function in a coordinate manner.

Although initially much scepticism surrounded the idea that CO could have a beneficial role *in vivo*, more and more publications have attributed positive effects to CO, whilst, the other products of HO activity (biliverdin, bilirubin and Fe), once thought of as waste, have also been found to play an important biological role lending credence to the claims of CO's beneficial role.

The study on CO biology has been restricted as compounds that could imitate the effect of CO were lacking. Scientists had to rely on upregulating HO activity or using exogenously applied CO gas but these methods are incable of directly addressing the basic role of CO in biological systems.

The discovery that transition metal carbonyls could be used to release CO *in vitro* and *in vivo* by our laboratory opened a new avenue into the potential of such molecules to be used to facilitate research and possibly in the future for therapeutic purposes (Johnson, Mann, Clark, Foresti, Green, & Motterlini 2003; Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b). The early indentified CO-RMs were not soluble in biological buffers but it was found that the coordination of a biological ligand onto the metal centre could make it more water-soluble. The first prototypic water-soluble CO-RM has been synthesised and was labelled CORM-3 (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). With the development of this molecule it was necessary to investigate its actions *in vitro* to establish an understanding of its biological function.

3.2 Objective

Test the prototypic water soluble CO-RM (CORM-3) to determine its toxicity in various cell lines, its effect on the HO system and its regulation of the inflammatory response inferred from TNF-α, nitrite and iNOS levels in macrophages.

3.3 Experimental Protocol

The following section details the protocol of experiments relative to the results of Chapter 1. The precise methods can be found in more detail in the Material and Methods chapter. Stock solutions of CORM-3 (10 mM) were freshly prepared before each experiment by dissolving the compound in pure distilled water. CORM-3 powder was layered with N_2 gas prior to returning it to storage in the freezer at -20°C.

3.3.1 Chemicals and reagents

Hemin (ferriprotoporphyrin IX chloride), tin protoporphyrin IX (SnPPIX) and biliverdin (biliverdin IX hydrochloride) were purchased from Porphyrin Products Inc (Logan, Utah, USA). Stock solutions of SnPPIX (10 mM) and biliverdin (10 mM) were prepared by dissolving the compounds in 0.1 M NaOH. The solutions were kept on ice and protected from light until use. Hemin (2 mM) was dissolved in 1 ml 0.1 M NaOH and the pH adjusted to 7.4 using phosphate buffered saline. Tricarbonylchloro(glycinato)ruthenium (II) ([$Ru(CO)_3Cl$(glycinate)] or CORM-3) was synthesised as previously described (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b; Foresti, Hammad, Clark, Johnson, Mann, Friebe, Green, & Motterlini 2004). CORM-3 was freshly prepared as a 10 mM stock solution in pure distilled water. Tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$ or CORM-2) was obtained from Sigma Aldrich and solubilized in dimethyl sulfoxide (DMSO) to obtain a 10 mM stock. The chemical structures of CORM-2 and CORM-3 are represented in Fig 1. Inactive forms of each compound (negative controls) were also used in some experiments and they were prepared as follows: CORM-3

was 'inactivated' (iCORM-3) by adding the compound to cell culture medium and leaving it for 18 h at 37 °C in a 5% CO_2 humidified atmosphere to liberate CO. The iCORM-3 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. In the case of CORM-2, the inactive form was $Ru(DMSO)_4Cl_2$ (iCORM-2), a molecule where the carbonyl groups have been replaced with DMSO (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). Lipopolysaccharide (LPS - *E.Coli* serotype 026:B6) was obtained from Sigma. Polyclonal antibodies against HO-1 were from Bioquote Ltd (York, UK) and polyclonal anti-iNOS antibodies were purchased from Santa Cruz Biotechnology Inc (Wembley, Middlesex, UK). Antibodies against β-actin were purchased from Abcam (Cambridge, UK). All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

3.3.2 Myoglobin Assay

To prepare a stock solution of CORM-3 for the myoglobin assay, 1 mg of CORM-3 was added to 100 µl of dH_2O to give a 34 mM stock. From this solution, 4, 8 and 12 mM working stocks were prepared. The myoglobin was warmed (37 °C), sodium dithionite (0.1 %) added and a 1 ml sample of the myoglobin measured for the deoxy-Mb curve. This 1 ml sample of deoxy-Mb was then bubbled with CO gas for 10 seconds to saturate the myoglobin and give a MbCO curve. Six cuvettes were then loaded into the spectrophotometer carousel each containing 1 ml of deoxy-Mb solution each. Five µl of 4, 8, and 12 mM CORM-3 working stocks were then added in duplicate to each cuvette. The resultant final concentrations of CORM-3 being 20 (from 4 mM), 40 (from 8 mM)

and 60 μM (from 12 mM). The myoglobin was then mixed gently using a pipette and layered with 500 μl of mineral oil. The samples were then read at set intervals for a set period of time.

3.3.3 Cell Culture

Murine RAW264.7 monocyte macrophages (European Collection of Cell Cultures - Salisbury, Wiltshire, UK) were cultured as per Material and Methods. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

3.3.4 Experimental Protocol

Macrophages were exposed for 24 h to LPS (1 μg/ml) in the presence or absence of CORM-3 or CORM-2 (10, 50 and 100 μM) and nitrite levels and iNOS protein expression were determined at the end of the incubation. In a similar set of experiments, 50 and 100 μM CORM-3 were added to cells 6 h after incubation with LPS and nitrite measured at 24 h. The production of nitrite was also assessed in cells exposed simultaneously to LPS and CORM-3 (10, 50 and 100 μM) at 0 h followed by subsequent additions of CORM-3 at 3 and/or 6 h. Experiments were repeated with the negative controls iCORM-3 and iCORM-2 to assess whether the effects observed were due to the CO liberated by the CO-RMs or caused by other components of the molecules. The effect of CO-RMs and their inactive counterparts on the haem oxygenase pathway was also investigated. Specifically, cells were treated for 6 h in the presence of 10, 50 and 100 μM CO-RMs and haem oxygenase activity as well as HO-1 protein expression determined. Furthermore, haem oxygenase activity was measured

in cells treated with LPS in the presence or absence of CORM-3. Haem oxygenase activity and HO-1 expression were also evaluated in cells pre-incubated with 1mM NAC prior to exposure to 100 μM CORM-3. To exclude the possibility that endogenously generated haem degradation products would exert potential anti-inflammatory effects, the experiments were also conducted in the presence of SnPPIX (10 μM), an inhibitor of haem oxygenase activity. Furthermore, we investigated the effect of the haem oxygenase pathway on nitrite production by pre-treating cells for 6 h with either hemin (10, 25 and 50 μM) or biliverdin (1, 5, 10 and 20 μM) before exposure to LPS for 24 h. Experiments were also conducted using SnPPIX, which was present both during the pre-incubation with hemin and the exposure to LPS. The effect of 10, 50 and 100 μM CORM-3 on the cellular redox status was assessed by measuring glutathione levels at 30 min, 2 h and 4 h after CORM-3 exposure.

3.3.5 Assay for Nitrite Levels

Nitrite levels were determined using the Griess method. The measurement of this parameter is widely accepted as indicative of NO production. Briefly, the medium from treated cells cultured in 24 well plates was removed and placed into a 96 well plate (50 μl per well). The Griess reagent was added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0 μM to 300 μM in cell culture medium).

3.3.6 Western Blot Analysis

Samples of RAW264.7 cells were analysed by Western immunoblot. Briefly, an equal amount of protein (30 μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Bioquote Ltd., York, UK) or with a polyclonal rabbit anti-iNOS antibody (Santa Cruz Biotechnology Inc, Insight biotechnology, Wembley, Middlesex, UK) (1:1000 dilution in Tris-buffered saline, pH = 7.4). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualised using an amplified alkaline phosphatase kit from Sigma (Extra-3A. To verify equal loading, samples were also probed with β-actin polyclonal antibodies (Abcam, Cambridge, U.K.).

3.3.7 Assay for Haem Oxygenase Activity

Haem oxygenase activity was determined in RAW264.7 cells after various treatments. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH = 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 μM). The reaction was conducted at 37 °C in the dark for 1 h and terminated by the addition of 1 ml chloroform; the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

3.3.8 Determination of cellular glutathione content

The 5,5'. dithiobis-(2-nitrobenzoic acid) colorimetric assay was used for the measurement of glutathione. Briefly, cells at the end of the incubation period were washed with PBS and 600 µl of a 2% (w/v) solution of 5-sulfosalicylic acid were added for cell lysis and deproteinization. The samples were centrifuged for 5 min at 10,000 × g and 500 µl aliquots were reacted with 500 µl of 5,5' dithiobis-(2-nitrobenzoic acid) solution (0.3 M sodium phosphate buffer,10 mM EDTA and 0.2 mM 5,5' dithiobis-(2-nitrobenzoic acid), freshly prepared) and after 5 min the absorbance was read at 412 nm (extinction coefficient was 14.3 mM^{-1} cm^{-1}). Positive and negative controls were obtained by incubating macrophages with 1 mM NAC, a precursor of glutathione, or 1 mM DL-buthionine-[S,R]sulfoximine (BSO), an inhibitor of glutathione biosynthesis.

3.3.9 Determination of TNF-α levels

The level of TNF-α present in each sample was determined using a kit commercially available from R&D Systems (Abingdon, UK). The assay was performed according to the manufacturers' instructions. Briefly, cell culture supernatants were collected immediately after the treatment and spun at 13,000 x g for 2 min to remove any particulates. The medium was added to a 96 well plate pre-coated with affinity purified polyclonal antibodies specific for mouse TNF-α. An enzyme-linked polyclonal antibody specific for mouse TNF-α was added to the wells and left to react for 2 h followed by a final wash to remove any unbound antibody-enzyme reagent. The intensity of the colour detected at 450 nm (correction wavelength 570 nm) was measured after addition of a substrate solution and was proportional to the amount of TNF-α produced.

3.3.10 Cell Viability

Alamar Blue

Cell metabolism was determined using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK). The assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised (blue) form to a reduced (red) form. The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control.

Trypan Blue

The status of the cell membrane was investigated using Trypan blue. The medium of treated cells is removed and the cells then washed with PBS 10X. Then a 1:1 solution of PBS (10X) and Trypan blue dye was added to cells. The dye permeates cell in which the membrane has been compromised. By counting the number of cell dyed compared to those which are not the number of viable cells can be determined.

Lactate Dehydrogenase

Damage of the phopholipid bi-layer of cells was investigated using an LDH assay kit and carried out according to the manufacturer's instructions (Roche, UK). The medium of treated cells is collected, spun to remove any cellular debris and then a reaction mixture containg an INT dye added. LDH activity reflects its release from the cell membrane and a loss of viability.

3.3.11 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$.

3.4 Results

3.4.1 CORM-3 Releases a Quantifiable Amount of CO using the Myoglobin Assay

Having synthesised CORM-3, a water soluble transition metal carbonyl, the first factor to establish was that it was capable of releasing CO. This was tested using the myoglobin assay technique (paragraph 2.4). Figure 3.1A shows that indeed CORM-3 converted deoxymyoglobin (deoxyMb) to MbCO. In this instance, there is little change from time zero to 60 min which means that CORM-3 releases almost instantly all of its available CO into the myoglobin solution. The saturated MbCO curve, using CO gas, represents the maximal CO capacity of the myoglobin stock prepared that specific day. This is also quantified by the MbCO value reported at the top of the page (~51 μM). Although the stock concentration of myoglobin is 66 μM we can only saturate it to ~51 - 54 μM using CO gas. This is because the myoglobin purchased from sigma is impure and comes as 90% myoglobin, therefore, the concentration of MbCO we measure having saturated the myoglobin with CO gas is the real concentration of our myoglobin stock (54 μM is indeed ~90% of the expected 66 μM). Importantly, this effect was consistent throughout all myoglobin assays.

Subsequent conversion of the MbCO curve to empirical data (Figure 3.1B) gives us an idea of the CO released in solution. CORM-3 released ~35 μM of CO instantly and then went on to release a maximal of ~40 μM CO after only 5 min. Thus, the kinetics of the reaction are instant and the half life very short. The overall CO release suggests that one carbonyl group per CORM-3 molecule is liberated which is in agreement with earlier tests (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b; Foresti, Hammad,

Clark, Johnson, Mann, Friebe, Green, & Motterlini 2004). The release of CO from CORM-3 was concentration dependent. Also 20 μM and 60 μM CORM-3 released CO in an approximately 1:1 ratio. Addition of CORM-3 to biological buffers results in CO release and leaves an inactive CORM-3 (iCORM-3) not capable of further CO release (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b; Foresti, Hammad, Clark, Johnson, Mann, Friebe, Green, & Motterlini 2004). This was demonstrated by using the myoglobin assay and showed that iCORM-3 was incapable of converting deoxyMb to MbCO.

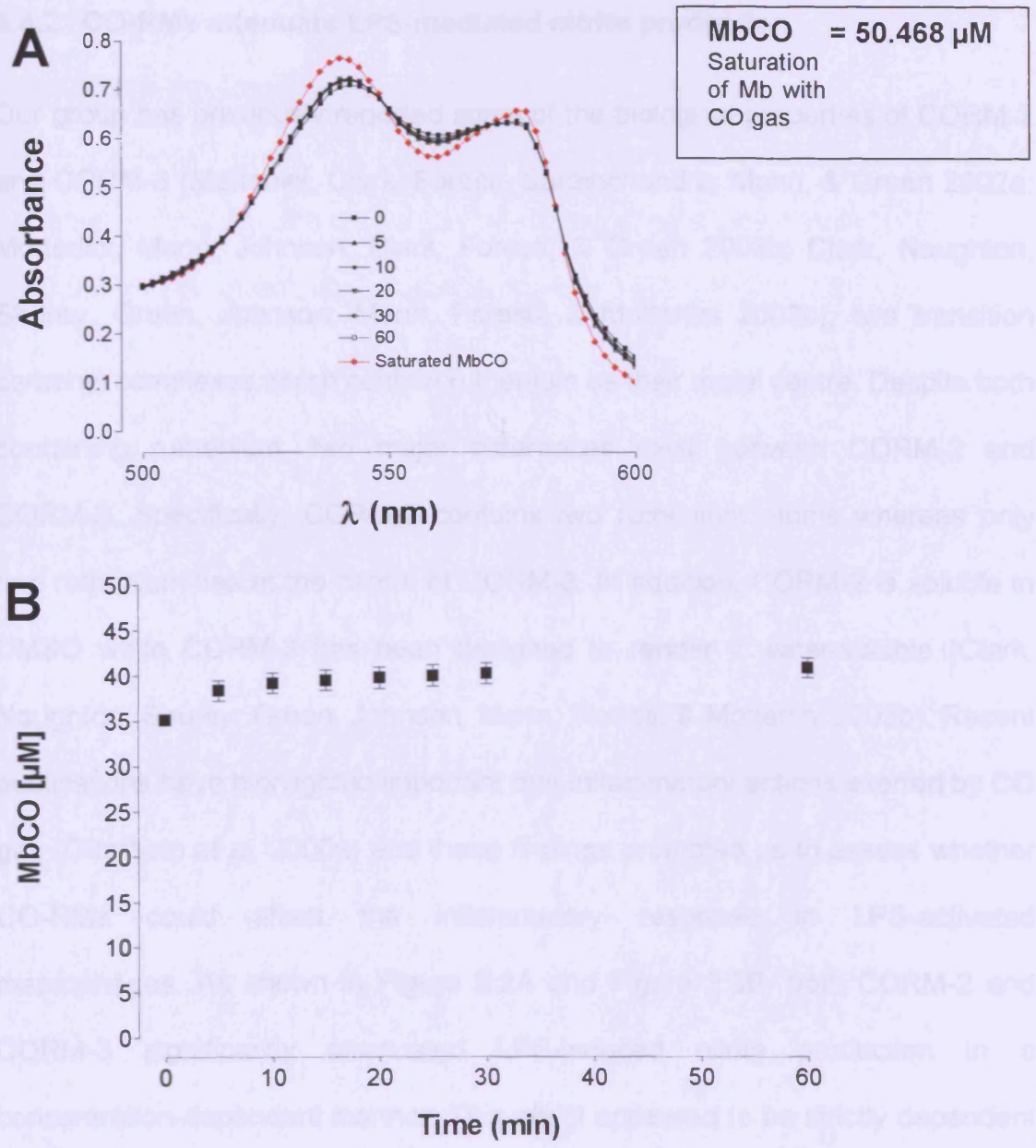


Figure 3.1:- CORM-3 releases a quantifiable amount of CO using the myoglobin assay.
(A) CORM-3 (40 µM) was added to a provisional 66 µM myoglobin solution the effective concentration is 51 µM as shown by the saturation with CO gas (see text on page 127 for details). The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-3 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

3.4.2 CO-RMs attenuate LPS-mediated nitrite production

Our group has previously reported some of the biological properties of CORM-2 and CORM-3 (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a; Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b; Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b), two transition carbonyl complexes which contain ruthenium as their metal centre. Despite both containing ruthenium, two major differences exist between CORM-2 and CORM-3. Specifically, CORM-2 contains two ruthenium atoms whereas only one ruthenium lies at the centre of CORM-3. In addition, CORM-2 is soluble in DMSO while CORM-3 has been designed to render it water-soluble (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). Recent publications have highlighted important anti-inflammatory actions exerted by CO gas (Otterbein et al. 2000a) and these findings prompted us to assess whether CO-RMs could affect the inflammatory response in LPS-activated macrophages. As shown in Figure 3.2A and Figure 3.3B, both CORM-2 and CORM-3 significantly attenuated LPS-induced nitrite production in a concentration-dependent manner. This effect appeared to be strictly dependent on the CO released by the compounds since neither iCORM-2 nor iCORM-3, which do not liberate CO, had any effect on nitrite levels. Interestingly, CORM-3 decreased nitrite even when added 6 h after LPS treatment (Figure 3.4A), suggesting that CO directly influences the production of NO by iNOS. Corroborating this idea, nitrite levels were lower in cells exposed to LPS and CORM-3 at 0 h followed by subsequent additions of CORM-3 at 3 and/or 6 h compared to treatment with LPS and CORM-3 at 0 h only (Figure 3.4B). Indeed, 100 μM CORM-3 delivered in multiple additions reduced nitrite to control levels.

It is important to note that cell viability was not affected by the concentrations of CO-RMs or the inactive forms used (Figure 3.2B, Figure 3.3B, Figure 3.5, Figure 3.6 and Figure 3.7), indicating that the results observed are not related to potential cytotoxicity of the compounds. These data suggest that CORM-2 and CORM-3 can modulate the production of nitrite by liberation of CO.

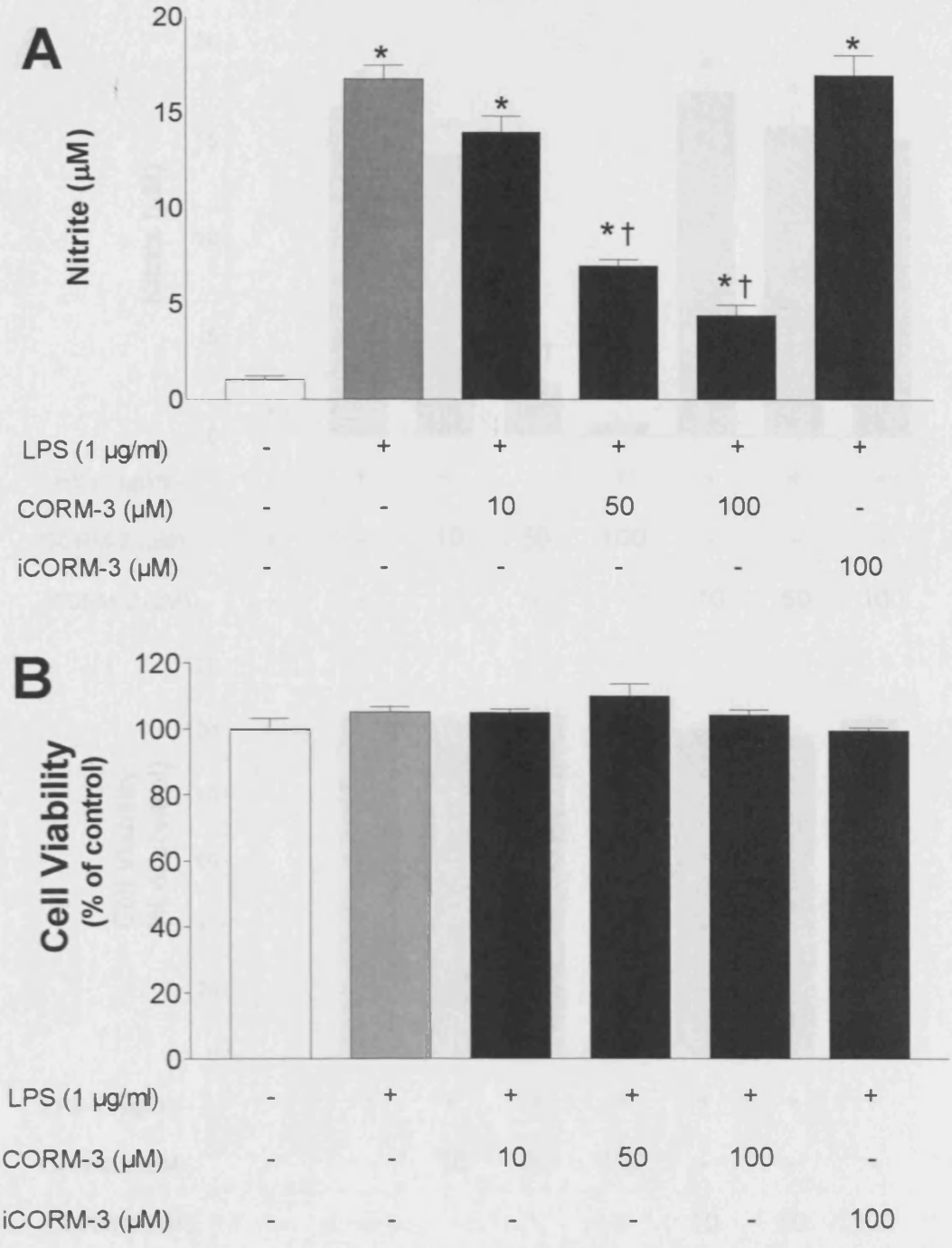


Figure 3.2:- Effect of CORM-3 on LPS-stimulated nitrite production and cell viability.

(A) RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-3 (10-100 μM) and nitrite production was assessed at 24 h. The inactive compound iCORM-3 (100 μM) was also used to determine the contribution of CO released by CORM-3 to the observed effect. Control cells were incubated with medium alone. (B) Cell viability was assessed 24 h after exposure of macrophages to 1 μg/ml LPS in the presence or absence of CORM-3 (10-100 μM) or iCORM-3 (100 μM). Viability was expressed as percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (white bars); † indicates $P < 0.05$ vs. LPS alone.

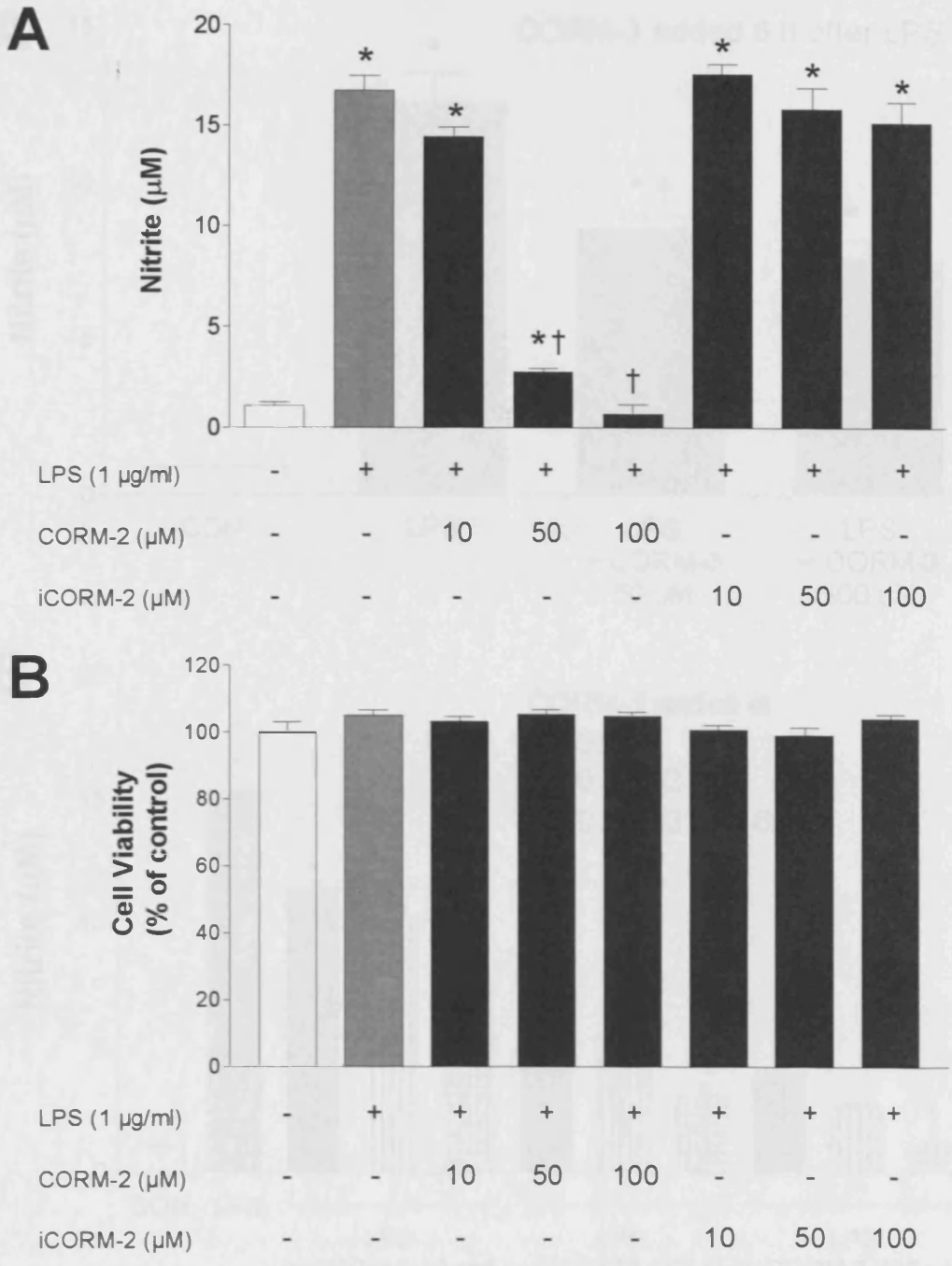


Figure 3.3:- Effect of CORM-2 on LPS-stimulated nitrite production and cell viability.
(A) RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-2 (10-100 μM) and nitrite production was assessed at 24 h. iCORM-2 (10-100 μM), an inactive compound that does not release CO, was also used as a negative control for CORM-2. Control cells were incubated with medium alone. (B) Cell viability was assessed 24 h after exposure of macrophages to 1 μg/ml LPS in the presence or absence of CORM-2 (10-100 μM) or iCORM-2 (10-100 μM). Viability was expressed as percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (white bars); † indicates $P < 0.05$ vs. LPS alone.

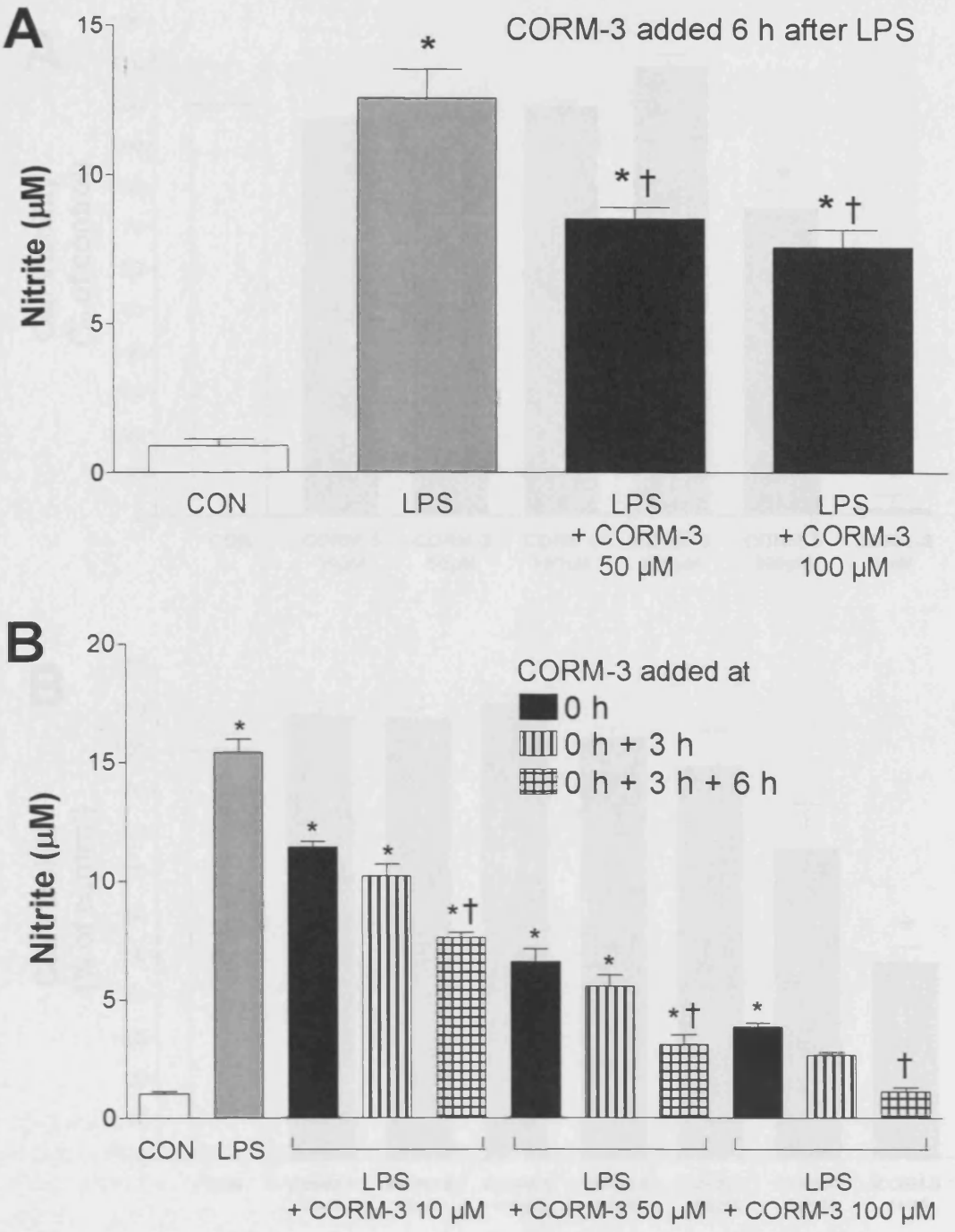


Figure 3.4:- Multiple additions of CORM-3 or its delivery after LPS challenge decrease nitrite production.
(A) RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence (0 h) or absence of CORM-3 (10-100 μM) and nitrite production was assessed at 24 h. In some experiments, CORM-3 was added also at 3 h after LPS (0 h + 3 h) or at 3 and 6 h after LPS (0 h + 3 h + 6 h). (B) Nitrite production was measured at 24 h in macrophages incubated with LPS alone or LPS followed 6 h later by a single addition of CORM-3 at 50 or 100 μM. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (CON); † indicates $P < 0.05$ vs. LPS alone.

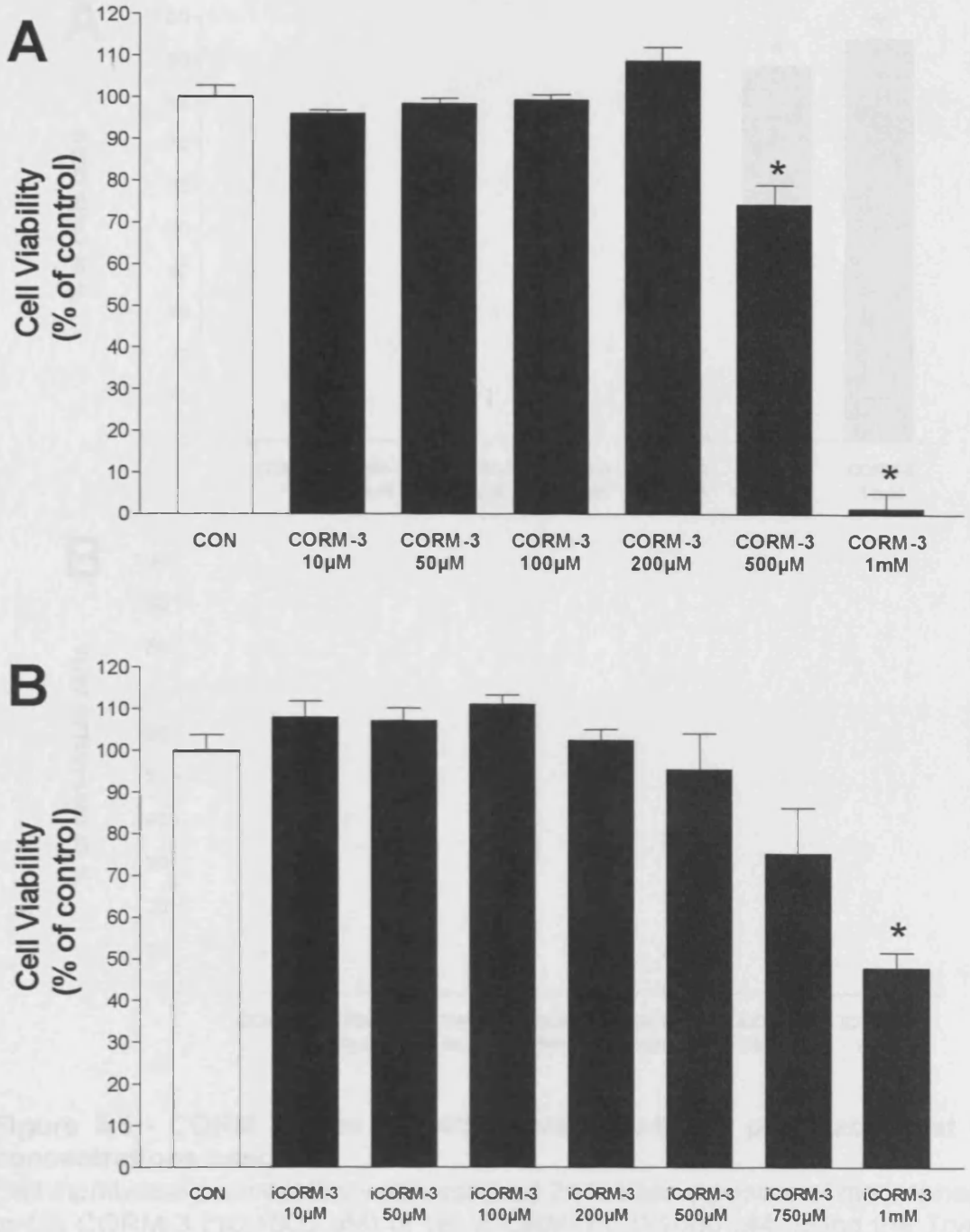


Figure 3.5:- CORM-3 does not affect cell metabolism at the concentrations used.

Cell metabolism was assessed 24 h after exposure of macrophages to (A) CORM-3 (10-1000 μM) or (B) iCORM-3 (10-1000 μM) using the Alamar blue assay. Viability was expressed as percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (CON).

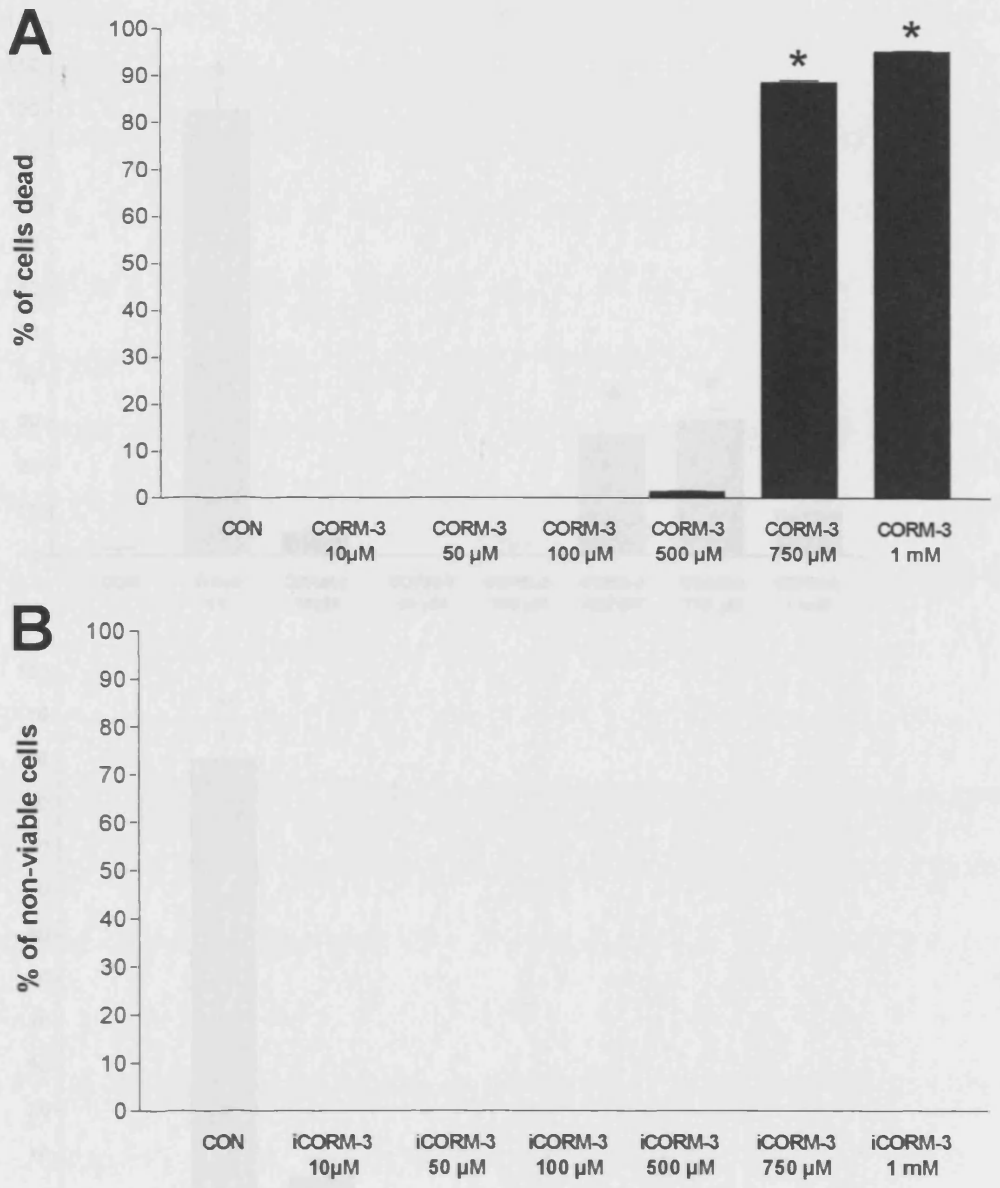


Figure 3.6:- CORM-3 does not affect cell membrane permeability at the concentrations used.

Cell membrane permeability was assessed 24 h after exposure of macrophages to (A) CORM-3 (10-1000 μM) or (B) iCORM-3 (10-1000 μM) using the Trypan blue assay. Viability was expressed as percentage stained cells compared to the total number of cells. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (CON).

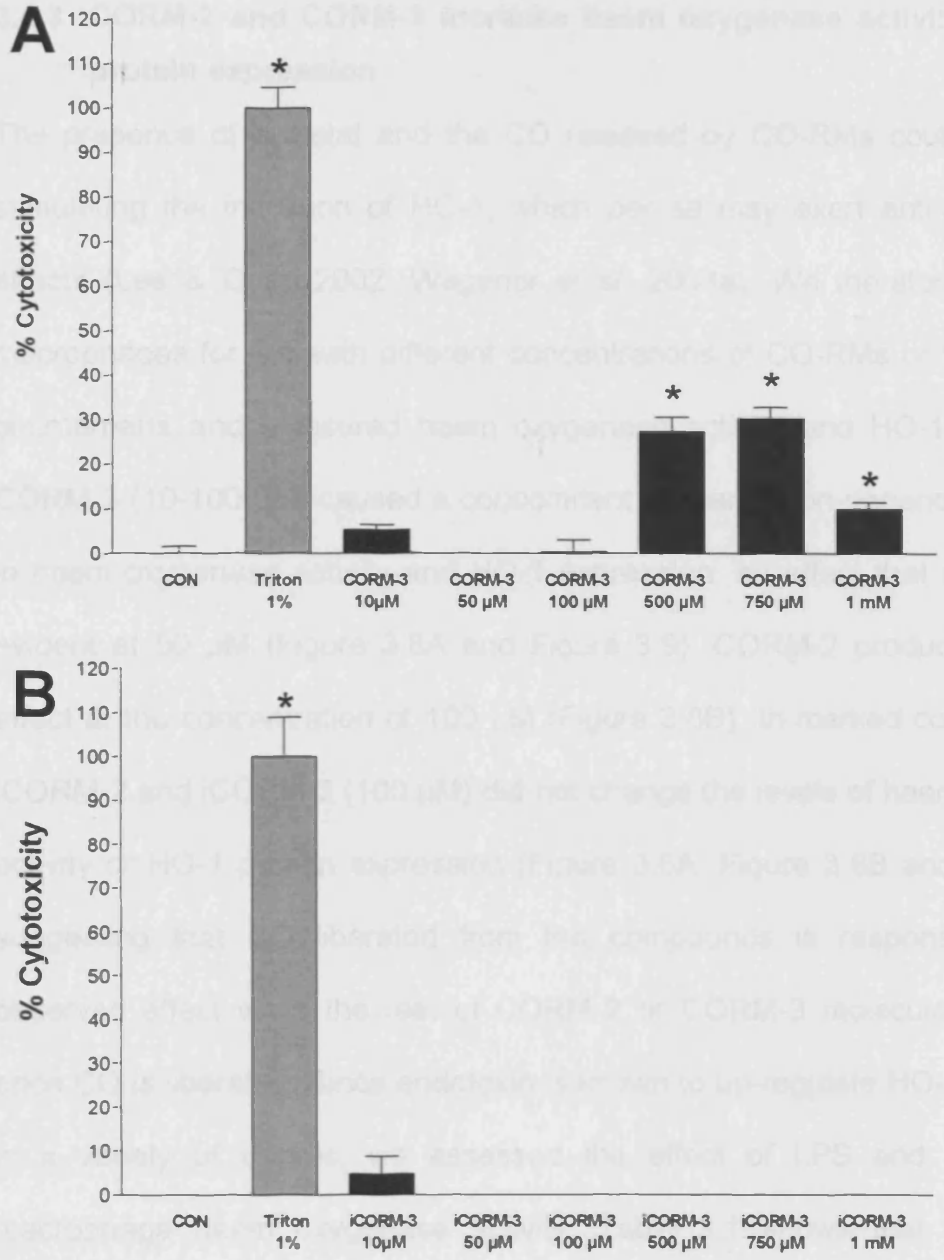


Figure 3.7:- CORM-3 does not affect LDH release at the concentrations used.

The release of LDH was assessed 24 h after exposure of macrophages to (A) CORM-3 (10-1000 µM) or (B) iCORM-3 (10-1000 µM) using the LDH assay. Viability was expressed as percentage of maximal damage (triton) and minmal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (CON).

3.4.3 CORM-2 and CORM-3 increase haem oxygenase activity and HO-1 protein expression

The presence of a metal and the CO released by CO-RMs could be factors stimulating the induction of HO-1, which *per se* may exert anti-inflammatory effects (Lee & Chau 2002; Wagener et al. 2003a). We therefore, incubated macrophages for 6 h with different concentrations of CO-RMs or their inactive counterparts and measured haem oxygenase activity and HO-1 expression. CORM-3 (10-100 μM) caused a concomitant concentration-dependent increase in haem oxygenase activity and HO-1 expression, an effect that started to be evident at 50 μM (Figure 3.8A and Figure 3.9). CORM-2 produced a similar effect at the concentration of 100 μM (Figure 3.8B). In marked constrast, both iCORM-2 and iCORM-3 (100 μM) did not change the levels of haem oxygenase activity or HO-1 protein expression (Figure 3.8A, Figure 3.8B and Figure 3.9) suggesting that CO liberated from the compounds is responsible for the observed effect while the rest of CORM-2 or CORM-3 molecules is inactive once CO is liberated. Since endotoxin is known to up-regulate HO-1 expression in a variety of tissues, we assessed the effect of LPS and CORM-3 on macrophage haem oxygenase activity. Table 3.1 shows that treatment of macrophages with LPS for 6 h resulted in a slight increase of haem oxygenase activity while the combination of LPS and CORM-3 did not differ from CORM-3 alone. In contrast, haem oxygenase was significantly increased after 24 h exposure to LPS and co-incubation of LPS with CORM-3 produced results similar to LPS alone. Previous reports have shown that induction of HO-1 suppresses the production of nitrite in macrophages stimulated with LPS (Turcanu, Dhouib, & Poindron 1998c). The above data therefore, could suggest

that CORM-2 and CORM-3 decrease nitrite production not because of their ability to release CO but rather by inducing HO-1 expression. To further investigate this possibility, we performed experiments in which macrophages were exposed to LPS and CORM-3 in the presence of SnPPIX (10 μM), a known inhibitor of haem oxygenase activity. We found that the nitrite production was not significantly different between macrophages stimulated by LPS and CORM-3 versus those treated with LPS and CORM-3 in the presence of SnPPIX (Figure 3.11). This suggests that HO-1 induction is not responsible for the decrease in nitrite levels observed in the presence of CORM-3 and substantiates the idea that CO released by CORM-3 mediates this anti-inflammatory effect. By using NAC, a thiol donor and glutathione precursor, we observed a complete suppression of HO-1 induction by CORM-3 (Figure 3.10), pointing to the potential of CO to modulate the redox status of the cell. We also examined the possibility that CORM-3 could affect the protein levels of iNOS, the inducible isoform of NO synthase which is stimulated by LPS and is accountable for the increased generation of NO during a variety of stress-related conditions. Figure 3.12 indicates that CORM-3 at different concentrations did not change the basal or LPS-stimulated expression of iNOS, emphasizing the possibility that CORM-3 interferes with the enzymatic activity of the protein.

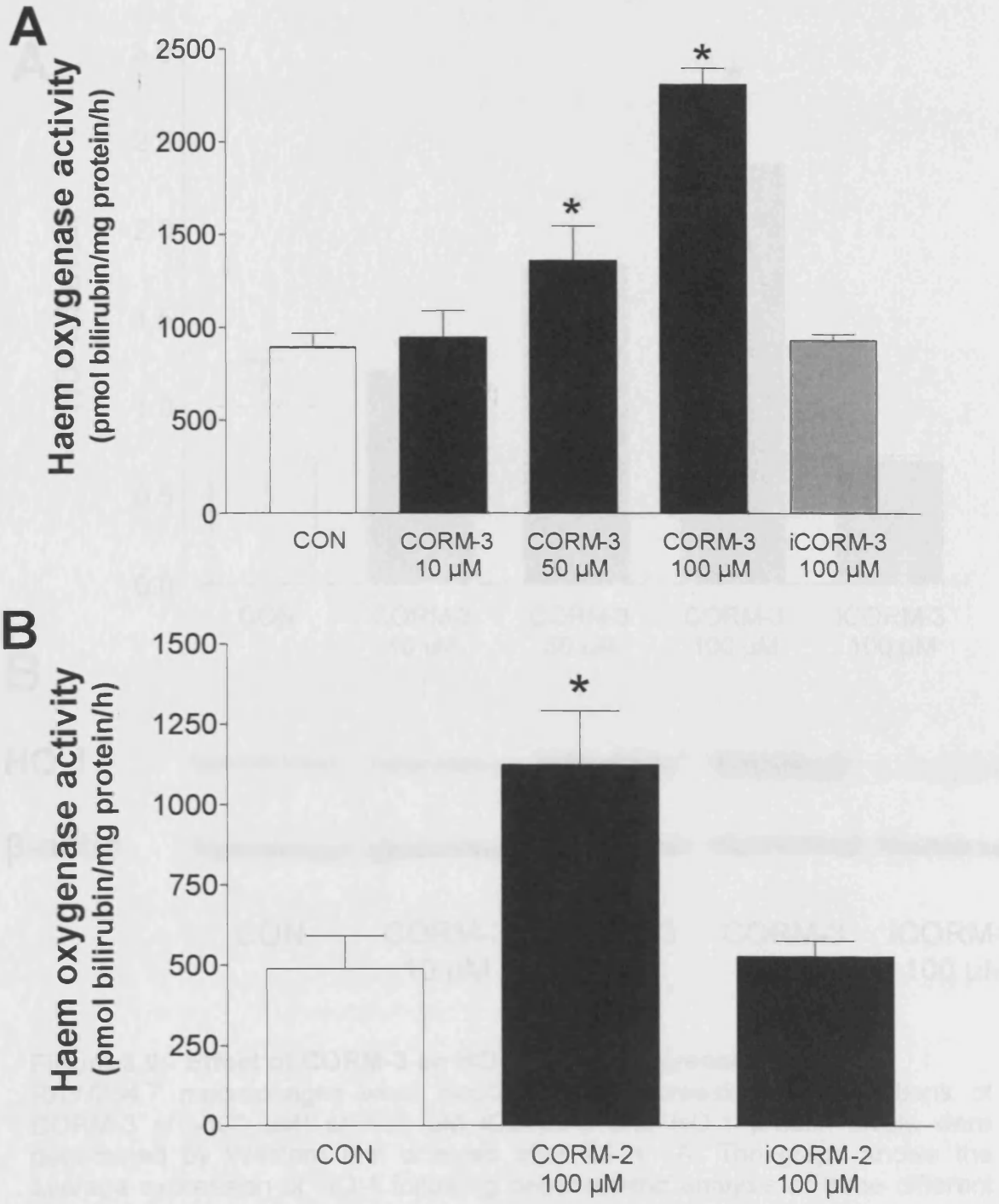


Figure 3.8:- Effect of CORM-3 and CORM-2 on haem oxygenase activity. (A) RAW264.7 macrophages were incubated with increasing concentrations of CORM-3 (10-100 µM) or iCORM-3 (100 µM). Haem oxygenase activity was determined 6 h after exposure to the different concentrations. (B) Haem oxygenase activity was measured in cells exposed to 100 µM CORM-2 or iCORM-2 for 6 h. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (CON).

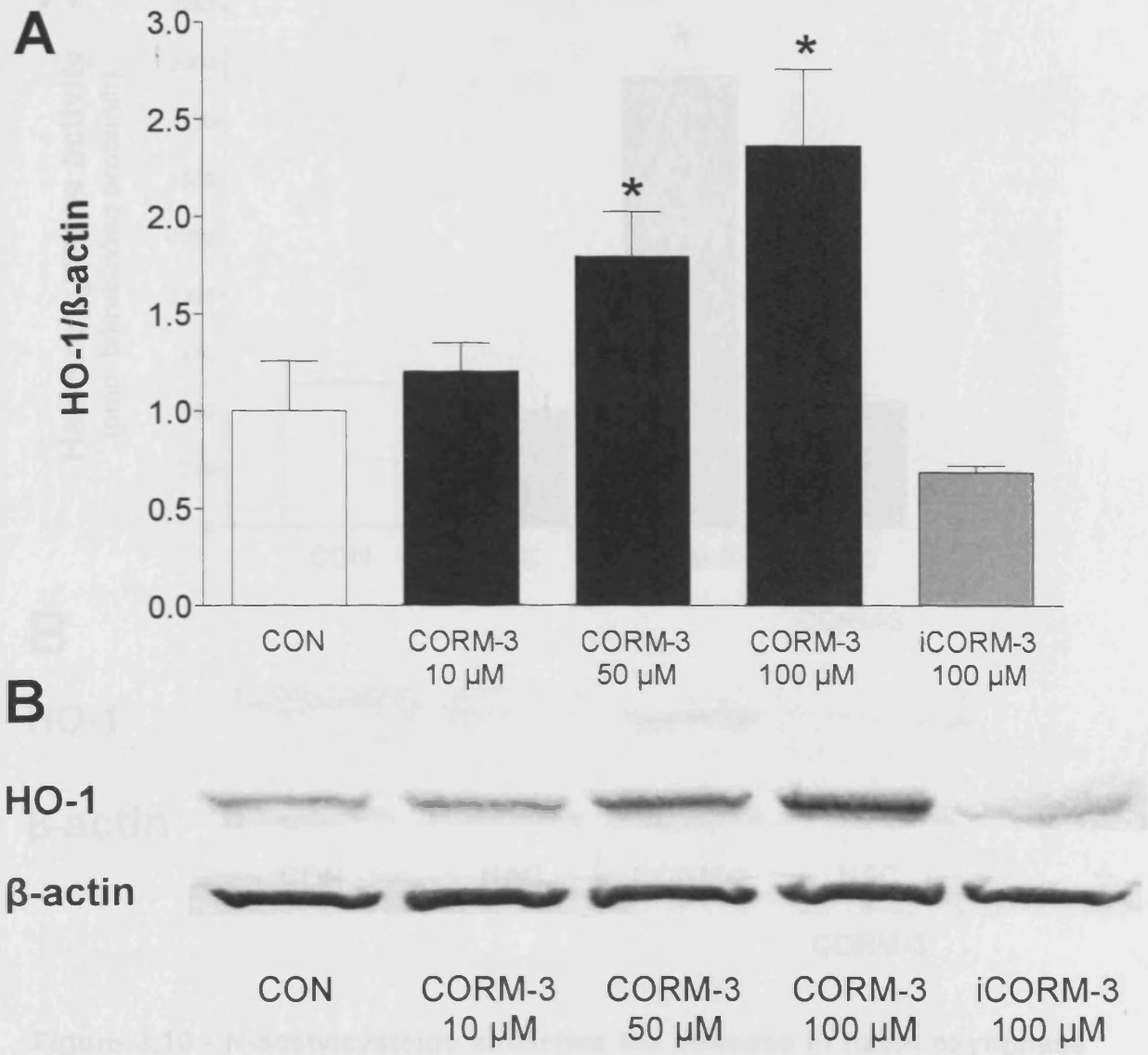


Figure 3.9:- Effect of CORM-3 on HO-1 protein expression.
RAW264.7 macrophages were incubated with increasing concentrations of CORM-3 (10-100 µM) or 100 µM iCORM-3 and HO-1 protein levels were determined by Western blot analysis after 24 h. (A) The graph shows the average expression of HO-1 following densitometric analysis of three different (B) blots from three independent experiments and one representative image is reported. β-actin was used as an internal control for equal loading. * indicates $P < 0.05$ vs. control (CON).

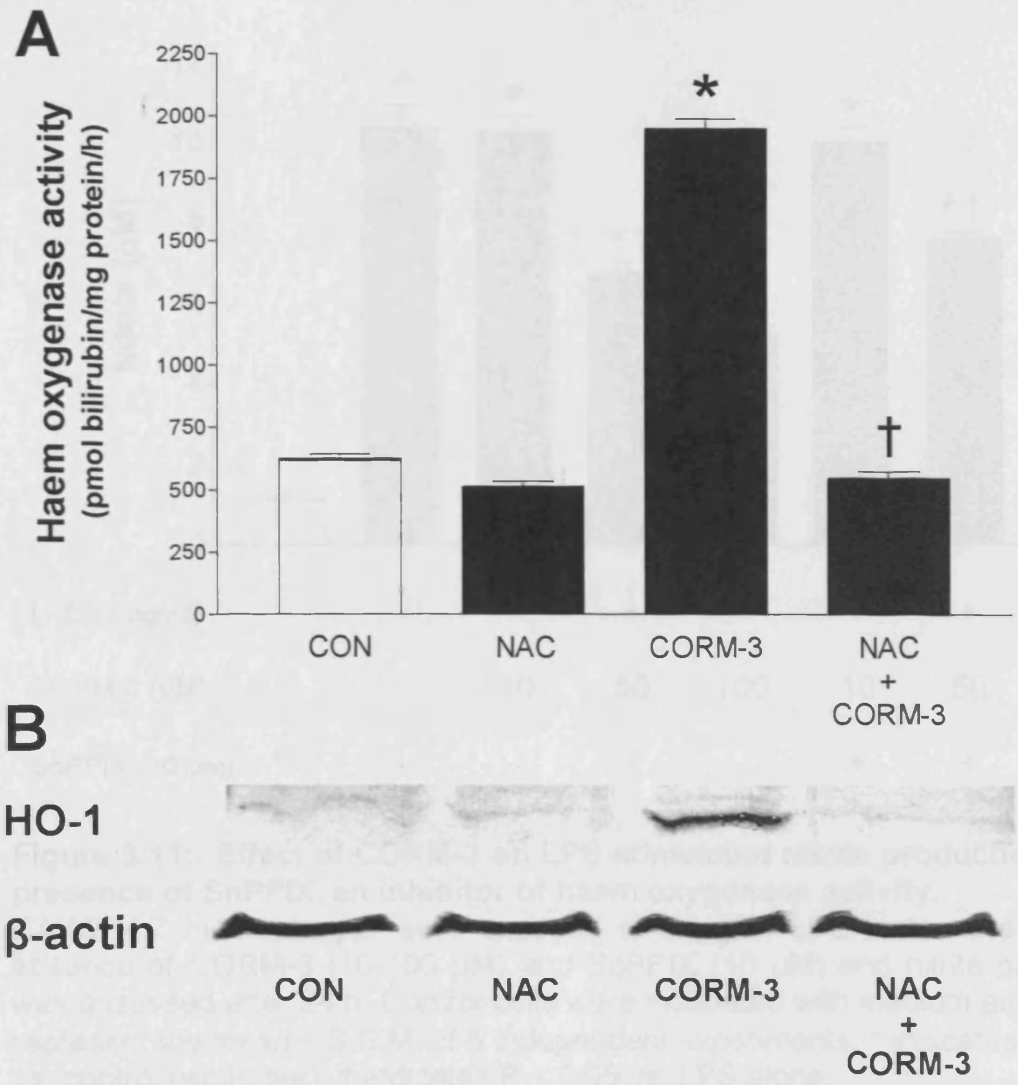


Figure 3.10:- N-acetylcysteine abolishes the increase in haem oxyegnase activity and HO-1 expression elicited by CORM-3.
RAW264.7 macrophages were incubated with 100 μM CORM-3 in the presence or absence of 1 mM NAC. (A) Haem oxygenase activity and (B) HO-1 expression were assessed after 6 h incubation. Data represent the mean ± S.E.M. of 6 independent experiments. Western blot image is representative of three different blots. β-actin was used as an internal control for equal loading. * indicates $P < 0.05$ vs. control (CON); † indicates $P < 0.05$ vs. CORM-3.

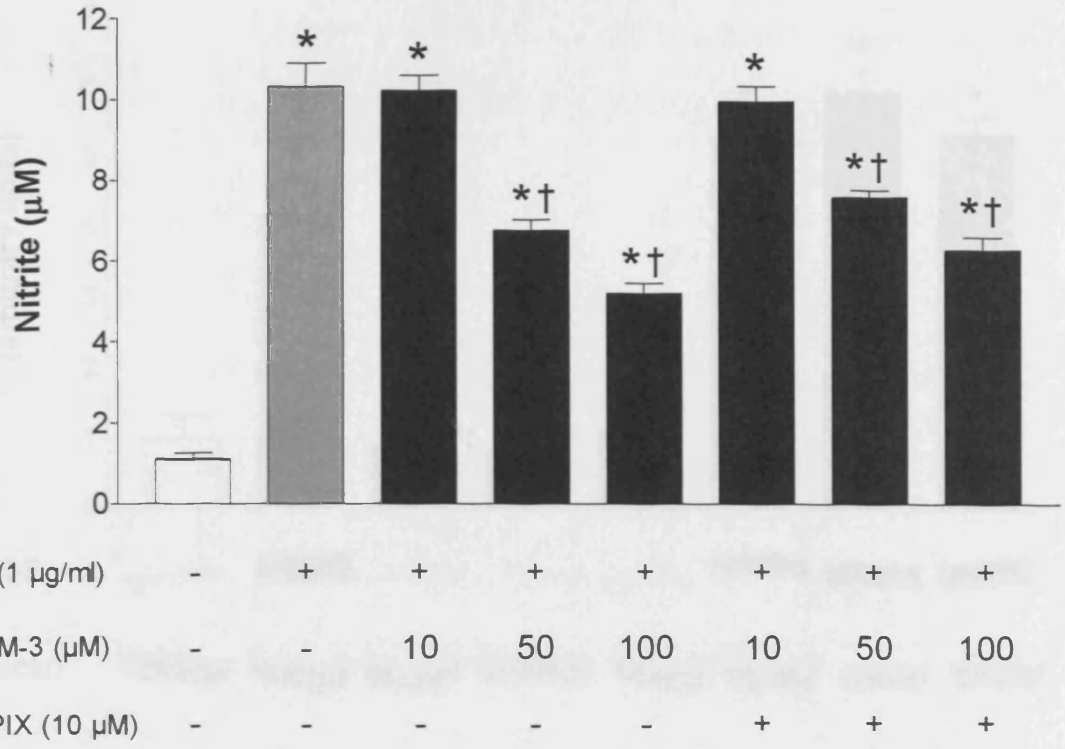


Figure 3.11:- Effect of CORM-3 on LPS-stimulated nitrite production in the presence of SnPPIX, an inhibitor of haem oxygenase activity.
RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-3 (10-100 μM) and SnPPIX (10 μM) and nitrite production was assessed after 24 h. Control cells were incubated with medium alone. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (white bar); † indicates $P < 0.05$ vs. LPS alone.

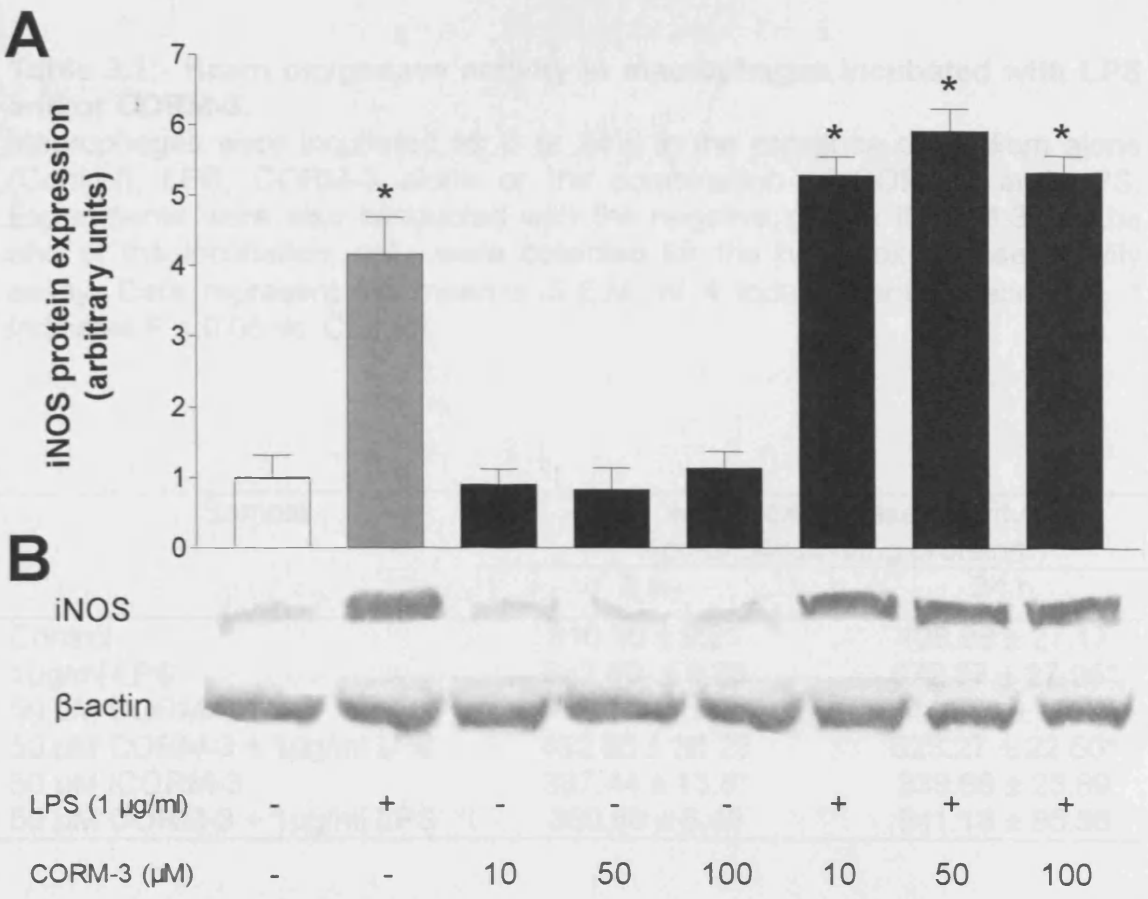


Figure 3.12:- Effect of CORM-3 on LPS-stimulated expression of inducible NO synthase (iNOS).
Macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-3 (10-100 μM) for 24 h and iNOS protein expression was examined by Western blot analysis. (A) The graph shows the average expression of iNOS protein following densitometric analysis of three different blots from three independent experiments and (B) one representative image is reported. β-actin was used as an internal control for equal loading. * indicates $P < 0.05$ vs. control.

Table 3.1:- Haem oxygenase activity in macrophages incubated with LPS and/or CORM-3.
Macrophages were incubated for 6 or 24 h in the presence of medium alone (Control), LPS, CORM-3 alone or the combination of CORM-3 and LPS. Experiments were also conducted with the negative control iCORM-3. At the end of the incubation, cells were collected for the haem oxygenase activity assay. Data represent the mean ± S.E.M. of 4 independent experiments. * indicates $P < 0.05$ vs. Control.

Sample	Haem oxygenase activity (pmol bilirubin/mg protein/h)	
	6 h	24 h
Control	310.70 ± 9.21	408.98 ± 27.17
1µg/ml LPS	342.60 ± 9.30	672.37 ± 27.95*
50 µM CORM-3	418.25 ± 16.3*	434.41 ± 8.07
50 µM CORM-3 + 1µg/ml LPS	452.85 ± 30.23	623.27 ± 22.50*
50 µM iCORM-3	387.44 ± 13.8*	339.66 ± 23.89
50 µM CORM-3 + 1µg/ml LPS	350.88 ± 6.49	541.18 ± 85.56

3.4.4 Effect of CORM-3 on cellular glutathione content

The results showing that NAC abolished the induction of HO-1 by CORM-3 suggested that CO exerted a degree of cellular oxidative stress. To examine this phenomenon more closely, we assessed the changes in glutathione levels during exposure to CORM-3 (10, 50 and 100 μM). As reported in Table 3.2, the glutathione content did not vary between control and CORM-3-treated cells following a 30 min incubation. However, after 2 h there was a significant decrease in glutathione in macrophages exposed to 100 μM CORM-3, while 10 and 50 μM CORM-3 only caused a slight change. Interestingly, at 4 h there was a complete recovery of the glutathione content with all three concentrations of CORM-3, suggesting that cells were rapidly reacting to the CO-mediated stress by stimulating *de novo* glutathione synthesis and thus re-establishing the normal endogenous redox balance. Since iCORM-3 did not affect glutathione (data not shown), we conclude that the effect caused by CORM-3 is due to CO liberated by the compound.

Table 3.2:- Glutathione content measured in macrophages incubated with CORM-3.

Macrophages were incubated for 30 min, 2 h or 4 h in the presence of medium alone (Control), or 10, 50 or 100 μM CORM-3 and glutathione content was assessed using a spectrophotometric assay. Experiments were also conducted with the negative control iCORM-3 without significant changes (data not shown). Data represent the mean ± S.E.M. of 3-5 independent experiments. * indicates $P < 0.05$ vs. Control.

Sample	Glutathione (% of control)		
	30 min	2 h	4h
Control	100±9.47	100±5.26	100±4.78
CORM-3 (10 μM)	110.02±1.50	97.94±2.62	102.51±2.35
CORM-3 (50 μM)	107.34 ±1.79	92.41±0.71	99.69±3.47
CORM-3 (100 μM)	109.97±1.04	81.17±5.66 *	106.26±3.15

3.4.5 CORM-3 attenuates LPS-mediated TNF- α production

Having established that CORM-3 could attenuate LPS-induced nitrite production, we investigated its effect on the generation of TNF-α, an additional marker of inflammation (Otterbein, Bach, Alam, Soares, Tao Lu, Wysk, Davis, Flavell, & Choi 2000a). As shown in Figure 3.13A, LPS caused a significant production of TNF-α in macrophages and the addition of 10 μM CORM-3 completely abolished this inflammatory response. Conversely, iCORM-3 did not change TNF-α levels, suggesting once again that CO is the active component of the CORM-3 molecule that elicits this effect. Considering that CORM-3 can induce HO-1 (see earlier results of Figure 3.8A and Figure 3.9), we assessed whether haem oxygenase activity was involved in the mechanisms modulating TNF-α production. Interestingly, treatment of macrophages with LPS in the presence of SnPPIX caused an even greater increase in TNF-α levels (Figure 3.13B), implying that endogenous haem oxygenase activity inhibits the production of this pro-inflammatory mediator. However, the amount of TNF-α was reduced to that of LPS alone when CORM-3 was added in the presence of LPS and SnPPIX (Figure 3.13B). These data suggest that CO plays an important role in the attenuation of LPS-induced TNF-α production, highlighting that inhibition of haem oxygenase activity also profoundly influences the levels of TNF-α.

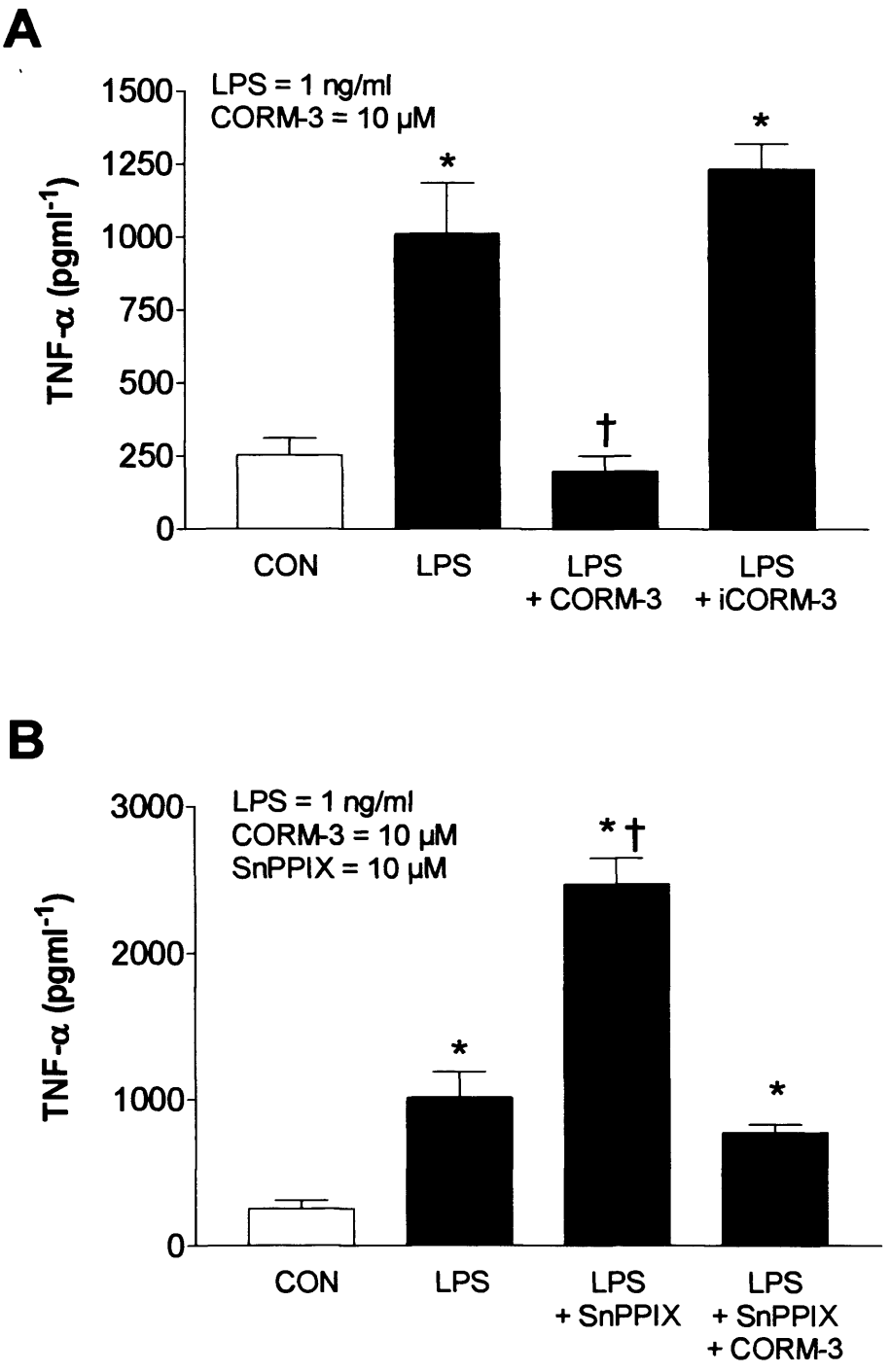


Figure 3.13:- CORM-3 modulates the production of TNF-α stimulated by LPS.

(A) Macrophages were exposed to 1 ng/ml LPS in the presence or absence of 10 µM CORM-3 or iCORM-3 and the level of TNF-α in the culture medium was measured at 24 h by a commercially available immunoassay. (B) TNF-α production was measured in macrophages stimulated by LPS in the presence or absence of CORM-3 and SnPPIX (10 µM). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ compared to control (CON); † indicates $P < 0.05$ vs. LPS alone.

3.4.6 Effect of hemin and bile pigments on nitrite production stimulated by LPS

To assess the contribution of haem oxygenase and endogenously-produced metabolites of its enzymatic activity (i.e. biliverdin/bilirubin, CO and ferrous iron) in regulating nitrite levels, we incubated cells with hemin, an inducer and substrate of haem oxygenase. As expected, macrophages exposed to hemin (10-50 μM) exhibited a concentration-dependent increase in haem oxygenase activity (Figure 3.14A). This effect was accompanied by a significant reduction in LPS-stimulated nitrite production (Figure 3.14B) and was partially reversed by inhibition of haem oxygenase activity with SnPPIX (Figure 3.15A). We also noted that stimulation of macrophages with LPS in the presence of SnPPIX resulted in an enhancement of nitrite levels compared to LPS alone (Figure 3.15A), suggesting that haem oxygenase exerts a negative feedback mechanism on NO generation during endotoxin treatment. As shown in Figure 3.15B, biliverdin (or bilirubin, data not shown) did not affect this response, suggesting that the nitrite reduction caused by hemin treatment is mediated by CO or possibly by degradation of the iNOS cofactor haem by haem oxygenase (Albakri & Stuehr 1996).

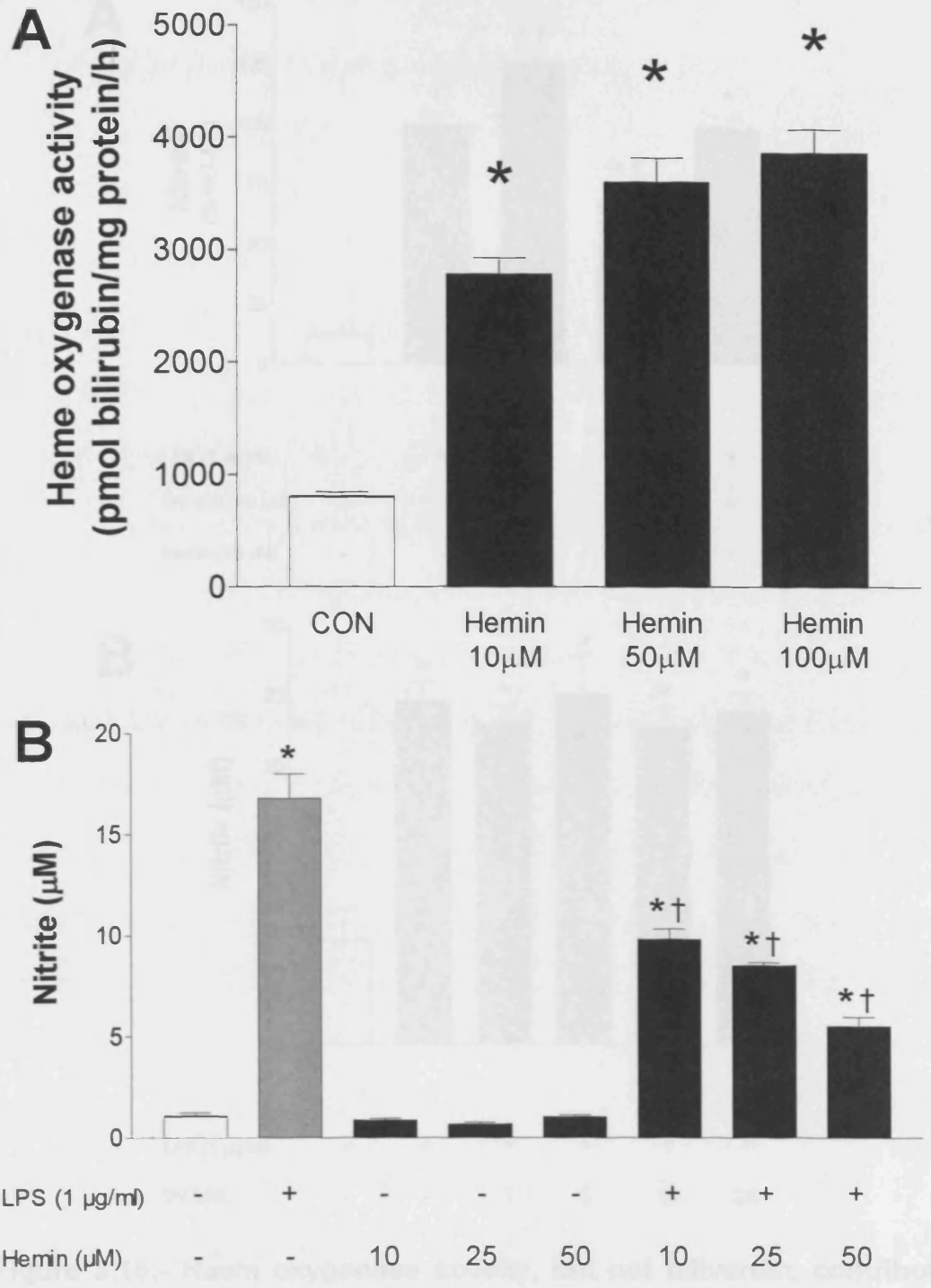


Figure 3.14:- Pre-incubation with hemin reduces LPS-stimulated nitrite production.

(A) Macrophages were treated with hemin (10-100 μM) for 6 h and haem oxygenase activity was measured at the end of the incubation as described. (B) Pre-treatment of macrophages with hemin (6 h) was followed by exposure to 1 μg/ml LPS and nitrite production was assessed after 24 h. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (white bars); † indicates $P < 0.05$ vs. LPS alone.

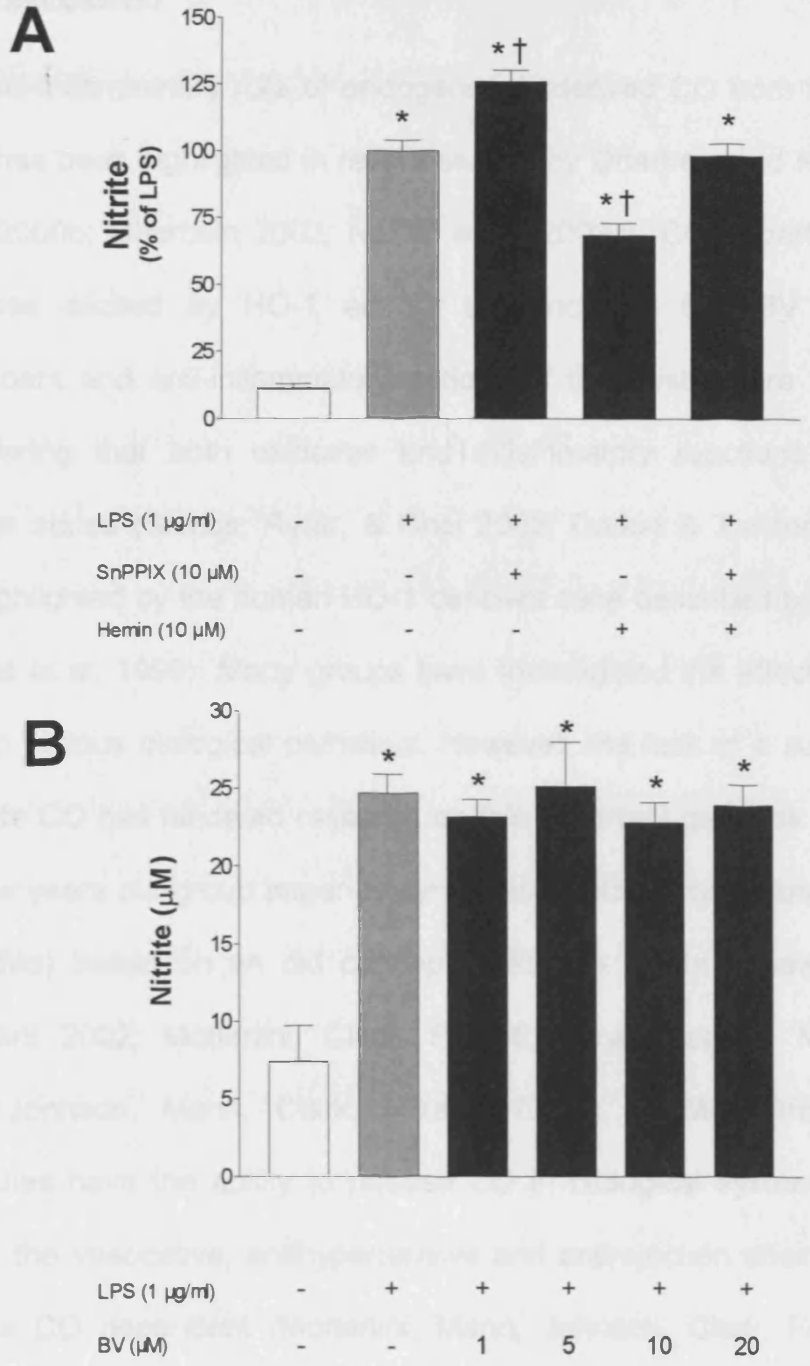


Figure 3.15:- Haem oxygenase activity, but not biliverdin, contributes to the reduction in nitrite production elicited by hemin.
(A) Macrophages were stimulated with 1 µg/ml LPS in the presence or absence of SnPPIX (10 µM) and nitrite production was measured at 24 h. In some experiments nitrite levels in the medium of macrophages pre-incubated with 10 µM hemin and SnPPIX prior to exposure to LPS were determined. (B) Nitrite levels were measured in medium of macrophages pre-incubated with biliverdin (1-20 µM) prior to exposure to LPS. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (white bars); † indicates $P < 0.05$ vs. LPS alone.

3.5 Discussion

The anti-inflammatory role of endogenously derived CO from the activation of HO-1 has been highlighted in recent studies by Otterbein and Nakao (Otterbein et al. 2000b; Otterbein 2002; Nakao et al. 2003c). CO is part of a concerted response elicited by HO-1 activity that includes BR, BV and iron. The antioxidant and anti-inflammatory actions of this system are vitally important considering that both oxidative and inflammatory reactions underlie many disease states (Slebos, Ryter, & Choi 2003; Dedon & Tannenbaum 2004). A fact highlighted by the human HO-1 deficient case described by Yachie in 1999 (Yachie et al. 1999). Many groups have investigated the effect of BR, BV and iron on various biological pathways. However, the lack of a suitable means to simulate CO has hindered research on this important gaseous mediator. In the last few years our group began to synthesise metal carbonyl-based compounds (CO-RMs) based on an old concept which we set in a new field (Mann & Motterlini 2002; Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002a;Johnson, Mann, Clark, Foresti, Green, & Motterlini 2003). These molecules have the ability to release CO in biological systems and we have shown the vasocative, antihypertensive and antirejection effects of these CO-RMs is CO dependent (Motterlini, Mann, Johnson, Clark, Foresti, & Green 2003b; Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b; Foresti, Hammad, Clark, Johnson, Mann, Friebe, Green, & Motterlini 2004). The success of these early studies led to continuing research to discover better and more biologically applicable CO-releasing agents.

CORM-3, the prototypic water soluble CO-RM, was found to elicit anti-inflammatory actions in an *in vitro* model of LPS-stimulated murine

macrophages. There was both a marked reduction in nitrite levels and TNF-α production, however, no effect on iNOS protein expression was observed. For comparison cells were also incubated with hemin, a potent inducer of HO-1, prior to LPS addition. This resulted in a decrease in nitrite similar to that witnessed with CORM-3. Interestingly incubating cells with either BV or BR had no effect. These data suggest an anti-inflammatory action for CORM-3 comparable to endogenously generated CO from HO-1.

The most likely explanation for the decrease in nitrite levels of macrophages exposed to LPS in the presence of CORM-3 is that NOS activity could be inhibited by CO. Indeed, CO gas has already been shown to potently inhibit the conversion of $_{L}$-arginine to NO and citrulline by neuronal and macrophage NOS. This is due to a conformational change at the active site of the enzyme elicited when CO binds the two haem moieties present in the active enzyme. This rationale is further supported by the fact CORM-3 did not affect iNOS expression and by the data showing nitrite levels were reduced further when CORM-3 was added after LPS treatment or in multiple additions when iNOS protein was already present. In addition, inactive CORM-3 did not affect nitrite implicating CO as the modulator of the observed effect. The inactive form of the compound also allows us to exclude the compound itself as an NO scavenger or that it interferes with NO generation.

Interestingly, CORM-3 has a half-life of 10.2 min in DMEM culture medium (as determined by the myoglobin assay), which is lessened in myoglobin itself, a more comparative reflection of the cellular milieu, to just 1-2 min. The intriguing question therefore remains how can CO elicit a significant reduction of nitrite 24 h after co-incubation with LPS? Cytotoxicity of CORM-3 can be excluded at the

concentrations used (10 – 100 μM) since neither the lactate dehydrogenase, Alamar blue or Trypan blue viability assays showed any reduction in overall cell viability. It is tempting to speculate that once CO is liberated it will tightly bind to cellular targets that preserve CO bioactivity over time in a manner similar to S-nitrosothiols (such as S-nitrosoalbumin and S-nitrosoglutathione) which are long-lived species acting as an apparent reservoir of NO bioactivity. Cellular targets that could act as a CO sink include haem dependent proteins or metalloproteins due to the high affinity of haem/metal prosthetic groups for CO. A plausible alternative for the results observed is that CO could modulate pathways influencing NO production by NOS.

A key anti-inflammatory action of CORM-3 was demonstrated by its significant attenuation of LPS-mediated TNF-α production. These data highlight two important issues: i) HO intrinsically modulates inflammation, since its inhibition during LPS exposure results in an enhancement of TNF-α productions and ii) endogenously applied CO carriers are an effect means to counteract HO pathway blockade. This data also confirms observations made by Otterbein and colleagues that low concentrations of CO gas inhibit the expression of LPS-induced TNF-α production *in vitro* (Sethi, Otterbein, & Choi 2002b). Further to this, a collaborative study we are involved in has shown that TNF-α production by isolated peripheral blood mononuclear cells stimulated with LPS in pigs treated with CORM-3 is significantly attenuated (data not published). The increase in HO-1 induction associated with the use of CORM-3 suggests that the release of CO causes some form of cellular stress. This concept was supported by results demonstrating that NAC completely abolished CORM-3 mediated HO-1 up-regulation and by measurements of total glutathione which

decreased significantly after 2 h but begun to increase at 4 h using 100 μM CORM-3. The fact 10 and 50 μM CORM-3 had no effect on glutathione suggests cellular stress is only associated with higher concentrations of CORM-3. This data correlates well with the finding that CORM-3 induced HO-1 at concentrations above 50 μM. The increase in HO-1, however, was not associated with the reduction in nitrite levels, possibly due to a limitation in substrate availability, since cells treated with CORM-3 and LPS in the presence of Sn-PPIX did not display any marked difference from those treated with just CORM-3 and LPS.

3.6 Conclusion

CORM-3 by way of CO suppresses LPS induced nitrite accumulation in a murine macrophage model of inflammation. The likely mechanism of action is via the direct inhibition of iNOS via CO binding and subsequent conformational distortion of the active site. This would explain the high iNOS expression and concomitant decrease in nitrite levels. CORM-3 would appear to be a valuable, non-toxic, tool in the elucidation of the role of CO in biological systems and more specifically has highlighted the action of CO in its anti-inflammatory regulation.

4 Chapter 4: Search and Characterisation of New CO-RMs; CO Release

4.1 Introduction

Having established that CORM-3 is bioactive *in vitro*, our chemist collaborator Professor Brian Mann (University of Sheffield) began to generate more CO-RMs based on the data and principles derived from CORM-3.

These metal carbonyls have different metal centres and biological ligands added. The very nature of metal carbonyl compounds allows for a great diversity of variants. Simple ligand substitution and changing the metal ion at the centre can completely alter the chemistry. This allows us to look at more biologically compatable metals such as iron. The original CO-RMs (CORM-2 and CORM-3) contained ruthenium, although, ruthenium was easy to manipulate chemically speaking in the developmenbt of early CO-RMs and is currently being used in a number of drugs and compounds currently being researched (Alessio et al. 2000; Alessio et al. 2004a; Alessio et al. 2004b) it was alluring to try and develop CO-RMs based on metals present in biology such as Fe and manganese (Mn) found commonly in enzymes (Groves 2003). Inherently this would have a number of desirable benefits: (i) make the CO-RM more biologically compatible and (ii) reduce any potential toxicity associated with the compound. There is also the potential for the body to assimilate the post CO release compound due to the presence of the iron, whereas, ruthenium does not occur in the body naturally and thus no system exists for its removal.

The overall aim being to try to generate a mixture of CO-RMs that could release large or small amounts of CO, that had various half-lives and were even less

likely to be toxic. Such variation is crucial not only from a research tool stand point but also in the ultimate goal of developing a therapeutic agent. For example, the constitutive action of HO may best be mimicked by a small CO release over a long period of time, whilst, an inducible HO response would require a large CO release in a short time. Such diversity could allow for a detailed examination of the role of CO in a variety of models, whilst, this variation would also be required in pathophysiological therapeutic treatment of chronic and acute conditions. The first step to establish with these new CO-RMs was which, if any, released CO. This was achieved using the myoglobin assay. The determination of CO released over time would also enable us to calculate the half life for each CO-RM.

4.2 Objective

Investigate the ability of newly synthesised CO-RMs to release CO using the myoglobin assay to determine new CO-RMs with the potential to forward the study.

4.3 Experimental Protocol

The following section details the protocol of specific experiments of which the general methods used can be found in the Material and Methods chapter. Further to this an exact list of all the CO-RMs tested can be found in the appendix (paragraph 11.1). Stock solutions of CO-RMs (10 mM) were freshly prepared before each experiment. CO-RMs were layered with N_2 gas prior to returning them to storage in the freezer at -20°C.

4.3.1 Myoglobin Assay

To prepare a stock of each CO-RM for the myoglobin assay 1 mg of each compound was added to 100 µl of dH_2O. From this initial stock, 4, 8 and 12 mM working stocks were prepared. The myoglobin was warmed (37 °C), sodium dithionite (0.1 %) added and a 1 ml sample of the myoglobin measured for the deoxy-Mb curve. This 1ml sample of deoxy-Mb was then bubbled with CO gas for 10 seconds to saturate the myoglobin and give a MbCO curve. Six cuvettes were then loaded into the spectrophotometer carousel with 1 ml of deoxy-Mb in each, 5 µl of 4, 8, and 12 mM CO-RM working stocks were then added in duplicate to each cuvette. The resultant final concentrations of CO-RM being 20 (from 4 mM), 40 (from 8 mM) and 60 µM (from 12 mM). The myoglobin was then mixed gently using a pipette and layered with 500 µl of mineral oil. The samples were then read at set intervals for a set period of time.

4.3.2 Inactivation of CO-RM

Very few of all the CO-RMs investigated could be inactivated in the same manner as CORM-3. It was found that by leaving both CORM-307 and CORM-308 in ethanol for 48 h they all but released their entire CO (as determined by the myoglobin assay). CORM-319 was also able to be inactivated by preparing 1 mg in 100 µl H_2O and leaving it to stand for 24 h in an open eppendorf after which the solution was bubbled with nitrogen (N_2) gas for 5 min to remove any CO trapped in solution.

4.4 Results

4.4.1 Overview

This section contains an assortment of CO-RMs tested using the myoglobin assay to give an idea of CO-RMs which release CO, and if so how much and how fast. The number of CO-RMs tested makes it impractical to show all the data collected; however, all the data is presented in a tabular form in the appendix (paragraph 11.1) for comparison.

4.4.2 CORM-303 Does not Release CO using the Myoglobin Assay

The spectrophotometric assay that detects the formation of MbCO from deoxy-Mb has been shown to be a reliable method for assessing the extent and kinetics of CO liberation from CO-RMs. A number of the CO-RMs tested displayed little (<5 μM) or no CO release. Figure 4.1A gives an example of such a CO-RM. There is no significant change in the myoglobin curve over time using CORM-303. In fact the curve retains the single peak characteristics of the deoxyMb curve unlike the double peak associated with the MbCO curve (Mb saturated with CO gas). There is no change in the curves characteristics over a 60 min period thus confirming its inability to release CO. Many similar chemically structured CO-RMs were also shown to be unable to release CO. The bioactivity of such CO-RMs was not investigated any further, although, they were not entirely useless since we could use them as a comparison to related molecules to try and identify components that may cause toxicity and maybe as inactive forms of closely related compounds.

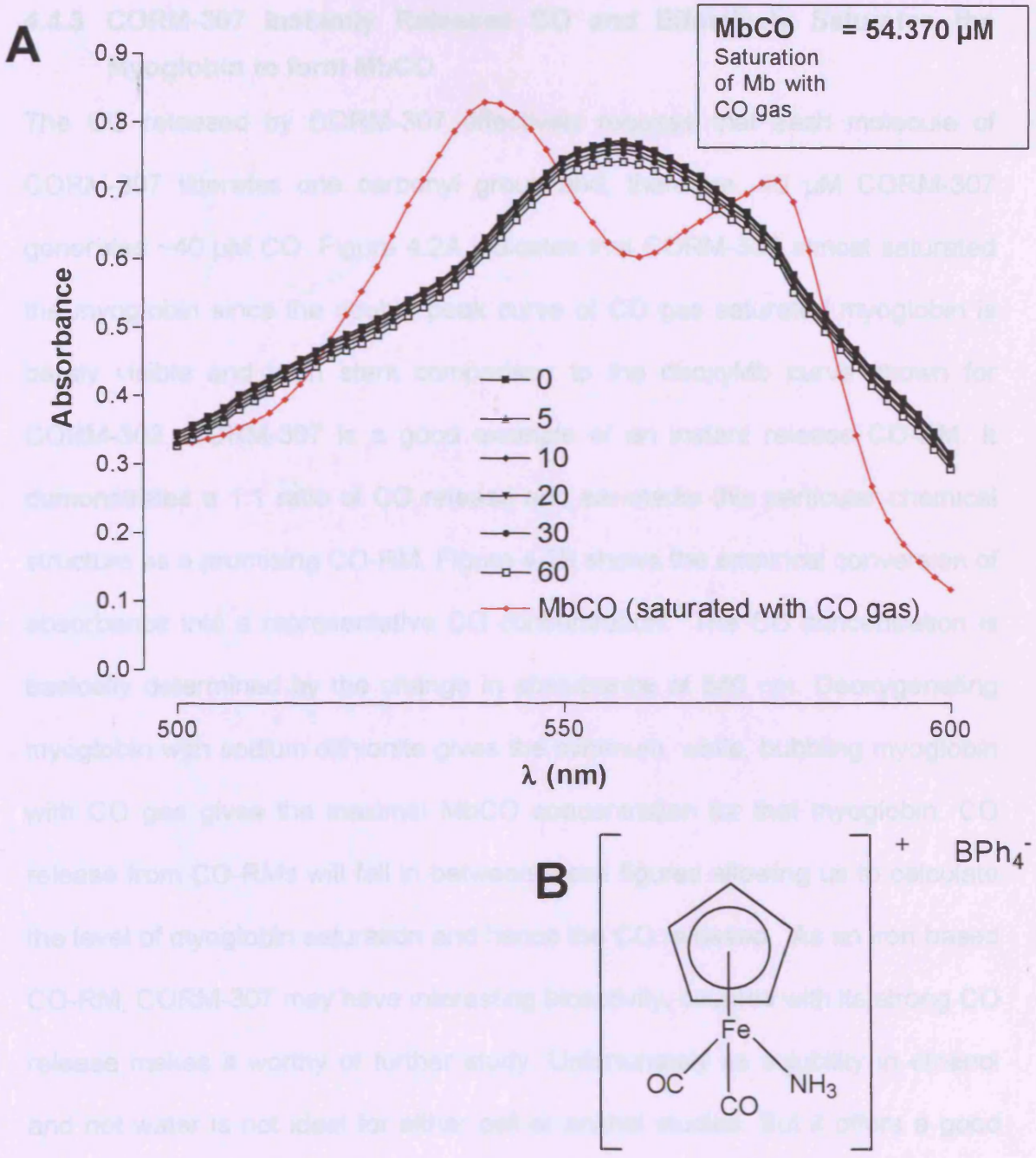


Figure 4.1:- Effect of CORM-303 on myoglobin: analysis of the conversion of Mb to MbCO.

(A) CORM-303 (40 µM) was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-303 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) Chemical structure of CORM-303.

4.4.3 CORM-307 Instantly Releases CO and Effectively Saturates the Myoglobin to form MbCO

The CO released by CORM-307 effectively requires that each molecule of CORM-307 liberates one carbonyl group and, therefore, 40 μM CORM-307 generates ~40 μM CO. Figure 4.2A indicates that CORM-307 almost saturated the myoglobin since the double peak curve of CO gas saturated myoglobin is barely visible and is in stark comparison to the deoxyMb curve shown for CORM-303. CORM-307 is a good example of an instant release CO-RM. It demonstrates a 1:1 ratio of CO release and ear-marks this particular chemical structure as a promising CO-RM. Figure 4.2B shows the empirical conversion of absorbance into a representative CO concentration. The CO concentration is basically determined by the change in absorbance at 540 nm. Deoxygenating myoglobin with sodium dithionite gives the minimum, while, bubbling myoglobin with CO gas gives the maximal MbCO concentration for that myoglobin. CO release from CO-RMs will fall in between these figures allowing us to calculate the level of myoglobin saturation and hence the CO released. As an iron based CO-RM, CORM-307 may have interesting bioactivity, coupled with its strong CO release makes it worthy of further study. Unfortunately its solubility in ethanol and not water is not ideal for either cell or animal studies. But it offers a good starting point for investigation of this class of CO-RMs.

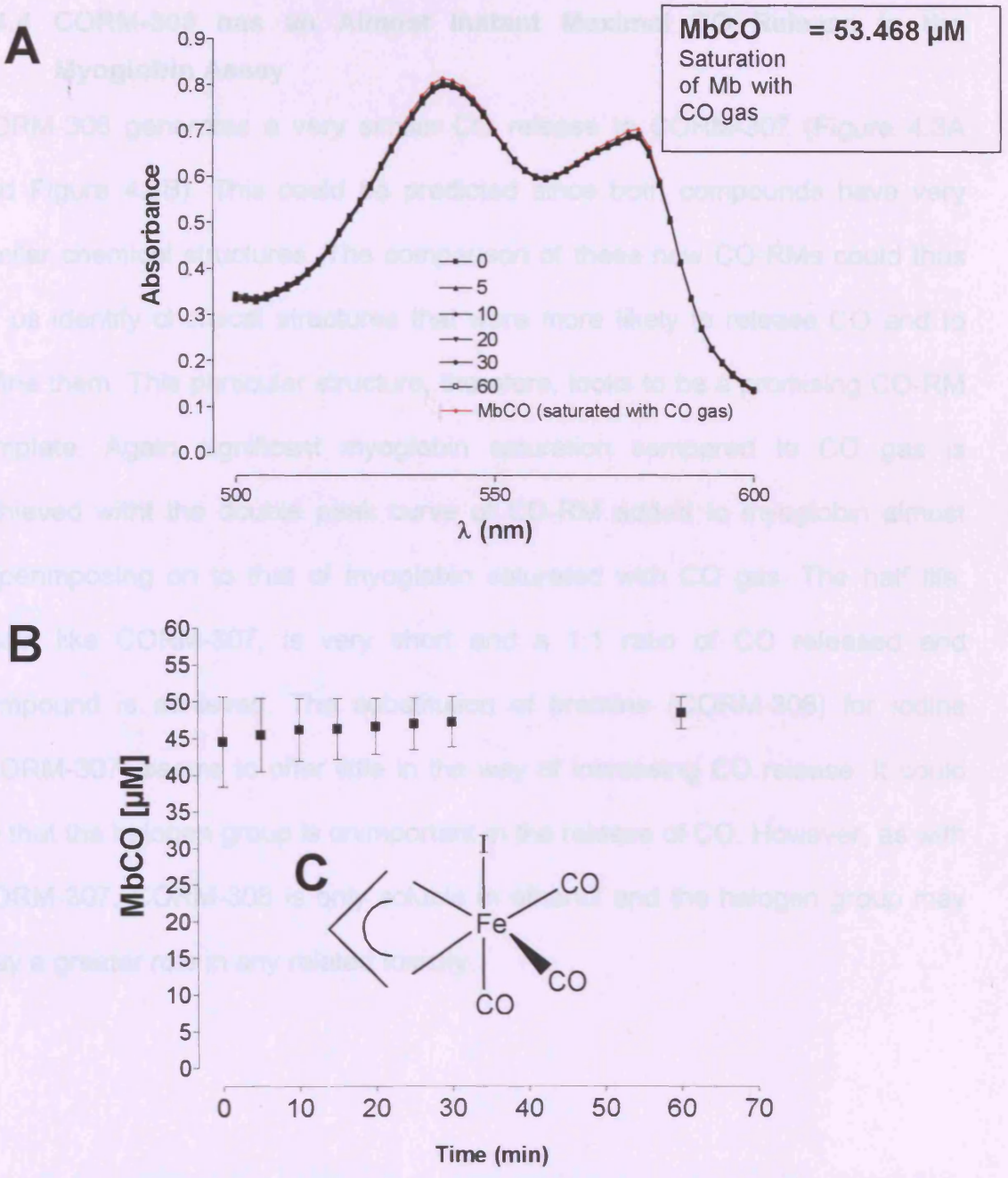


Figure 4.2:- Effect of CORM-307 on myoglobin CO saturation.
(A) CORM-307 (40 µM) was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-307 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution. (C) Chemical structure of CORM-307.

4.4.4 CORM-308 has an Almost Instant Maximal CO Release in the Myoglobin Assay

CORM-308 generates a very similar CO release to CORM-307 (Figure 4.3A and Figure 4.3B). This could be predicted since both compounds have very similar chemical structures. The comparison of these new CO-RMs could thus let us identify chemical structures that were more likely to release CO and to refine them. This particular structure, therefore, looks to be a promising CO-RM template. Again significant myoglobin saturation compared to CO gas is achieved witht the double peak curve of CO-RM added to myoglobin almost superimposing on to that of myoglobin saturated with CO gas. The half life, again like CORM-307, is very short and a 1:1 ratio of CO released and compound is achieved. The substitution of bromine (CORM-308) for iodine (CORM-307) seems to offer little in the way of increasing CO release. It could be that the halogen group is unimportant in the release of CO. However, as with CORM-307, CORM-308 is only soluble in ethanol and the halogen group may play a greater role in any related toxicity.

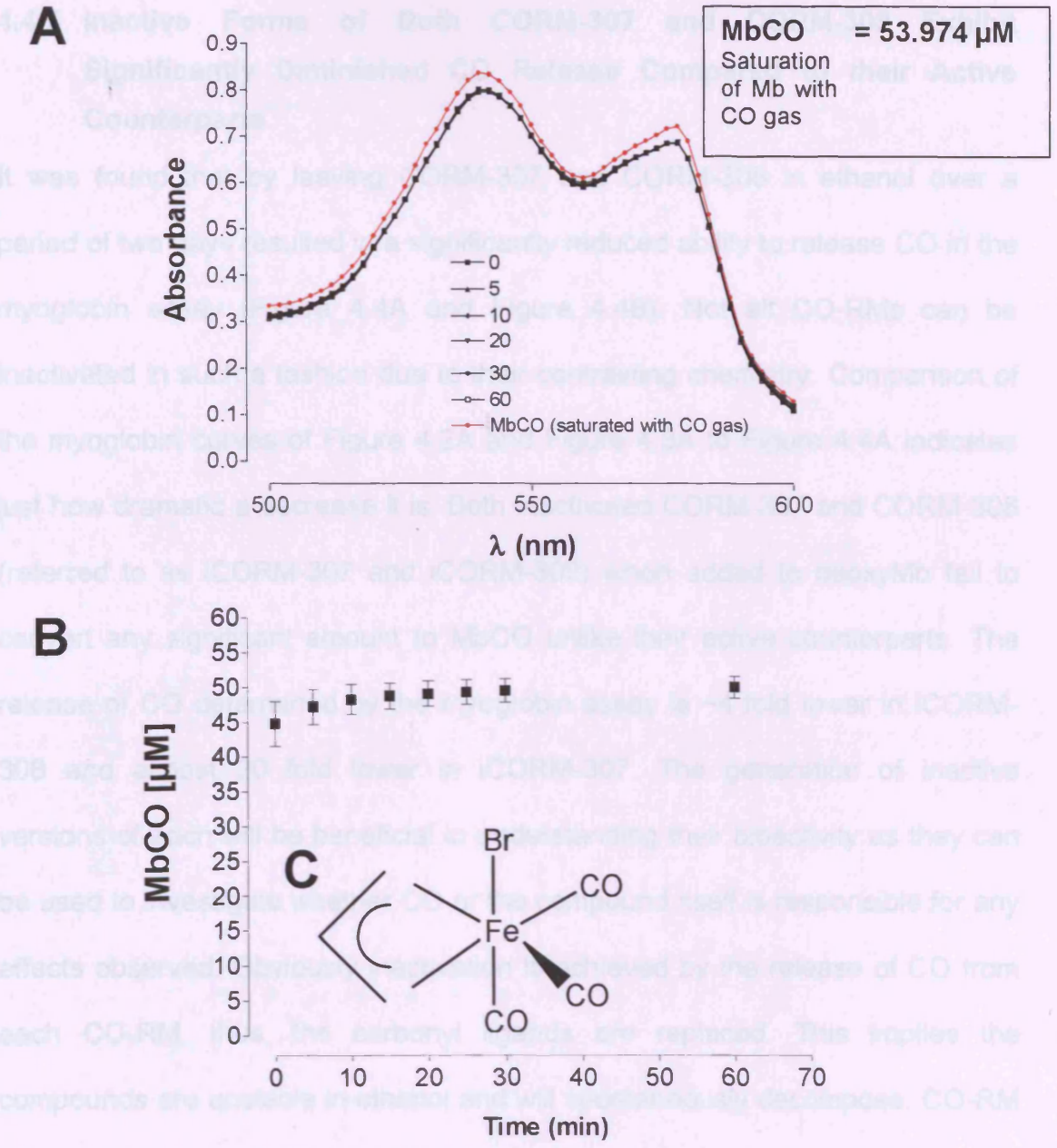


Figure 4.3:- Effect of CORM-308 on myoglobin: analysis of the conversion of Mb to MbCO.

(A) CORM-308 (40 µM) was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-308 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution. (C) Chemical structure of CORM-308.

4.4.5 Inactive Forms of Both CORM-307 and CORM-308 Exhibit Significantly Diminished CO Release Compared to their Active Counterparts

It was found that by leaving CORM-307 and CORM-308 in ethanol over a period of two days resulted in a significantly reduced ability to release CO in the myoglobin assay (Figure 4.4A and Figure 4.4B). Not all CO-RMs can be inactivated in such a fashion due to their contrasting chemistry. Comparison of the myoglobin curves of Figure 4.2A and Figure 4.3A to Figure 4.4A indicates just how dramatic a decrease it is. Both inactivated CORM-307 and CORM-308 (referred to as iCORM-307 and iCORM-308) when added to deoxyMb fail to convert any significant amount to MbCO unlike their active counterparts. The release of CO determined by the myoglobin assay is ~4 fold lower in iCORM-308 and almost 20 fold lower in iCORM-307. The generation of inactive versions of each will be beneficial in understanding their bioactivity as they can be used to investigate whether CO or the compound itself is responsible for any effects observed. Obviously inactivation is achieved by the release of CO from each CO-RM, thus, the carbonyl ligands are replaced. This implies the compounds are unstable in ethanol and will spontaneously decompose. CO-RM stability is another important issue to consider in the long term goal of a pharmamceutical viable compound since storage of the drug prior to treatment is essential.

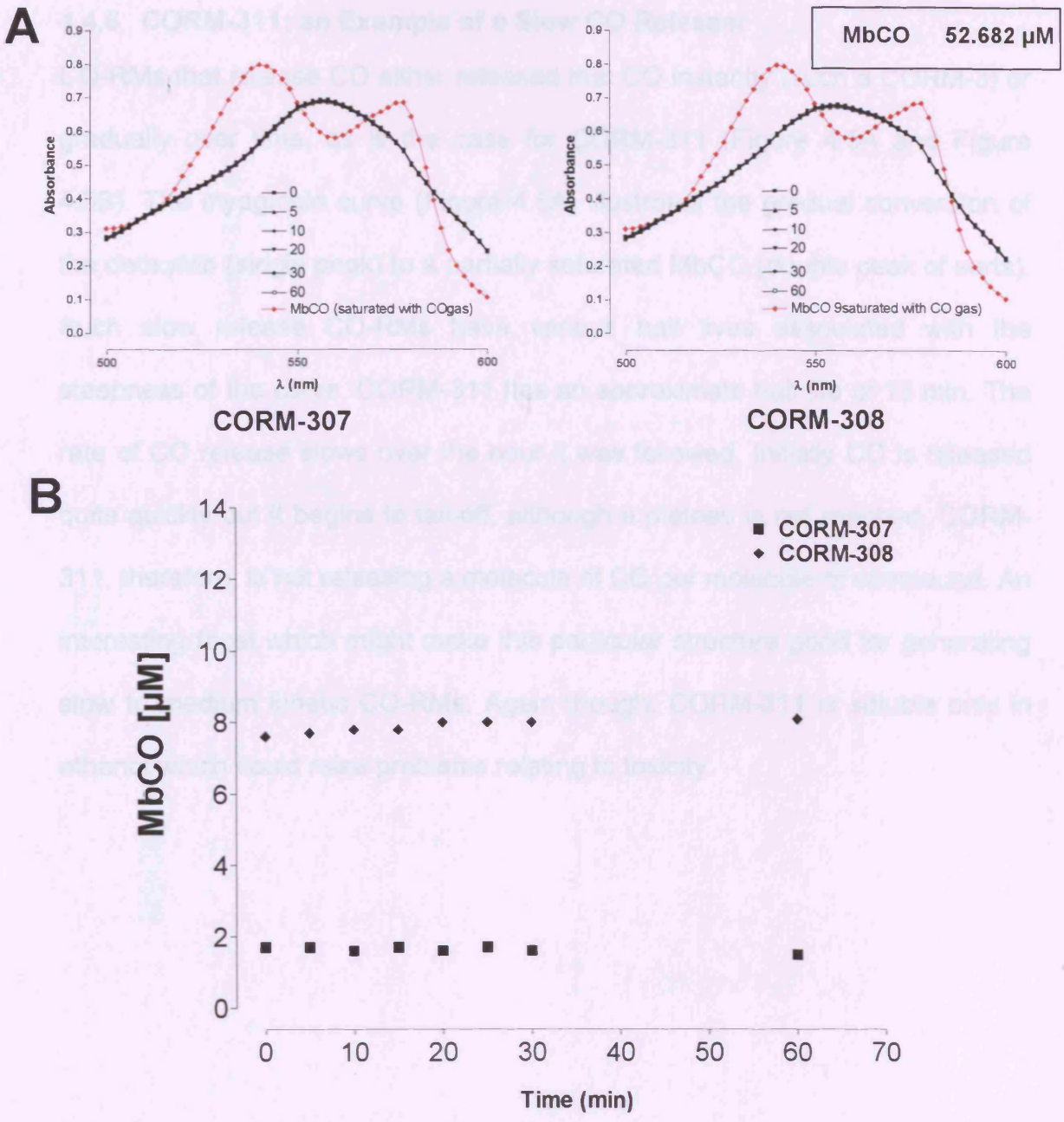


Figure 4.4:- Inactive forms of both CORM-307 and CORM-308 exhibit severely diminished CO release compared to their active counterparts.
Prior to testing with the myoglobin assay, both CORM-307 and CORM-308 stocks were prepared in ethanol and left to stand for 48 h in an open eppendorf to inactivate. Subsequently (A) iCORM-307 or iCORM-308 (40 µM) were added to a 66 µM myoglobin solution. Readings of any conversion of deoxyMb to MbCO were taken every 5 min for the first 30 min and then at 1h time points. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

4.4.6 CORM-311: an Example of a Slow CO Releaser

CO-RMs that release CO either released that CO instantly (such a CORM-3) or gradually over time, as is the case for CORM-311 (Figure 4.5A and Figure 4.5B). The myoglobin curve (Figure 4.5A) illustrates the gradual conversion of the deoxyMb (single peak) to a partially saturated MbCO (double peak of sorts). Such slow release CO-RMs have various half lives associated with the steepness of the curve. CORM-311 has an approximate half life of 15 min. The rate of CO release slows over the hour it was followed. Initially CO is released quite quickly but it begins to tail-off, although a plateau is not reached. CORM-311, therefore, is not releasing a molecule of CO per molecule of compound. An interesting facet which might make this particular structure good for generating slow to medium kinetic CO-RMs. Again though, CORM-311 is soluble only in ethanol which could raise problems relating to toxicity.

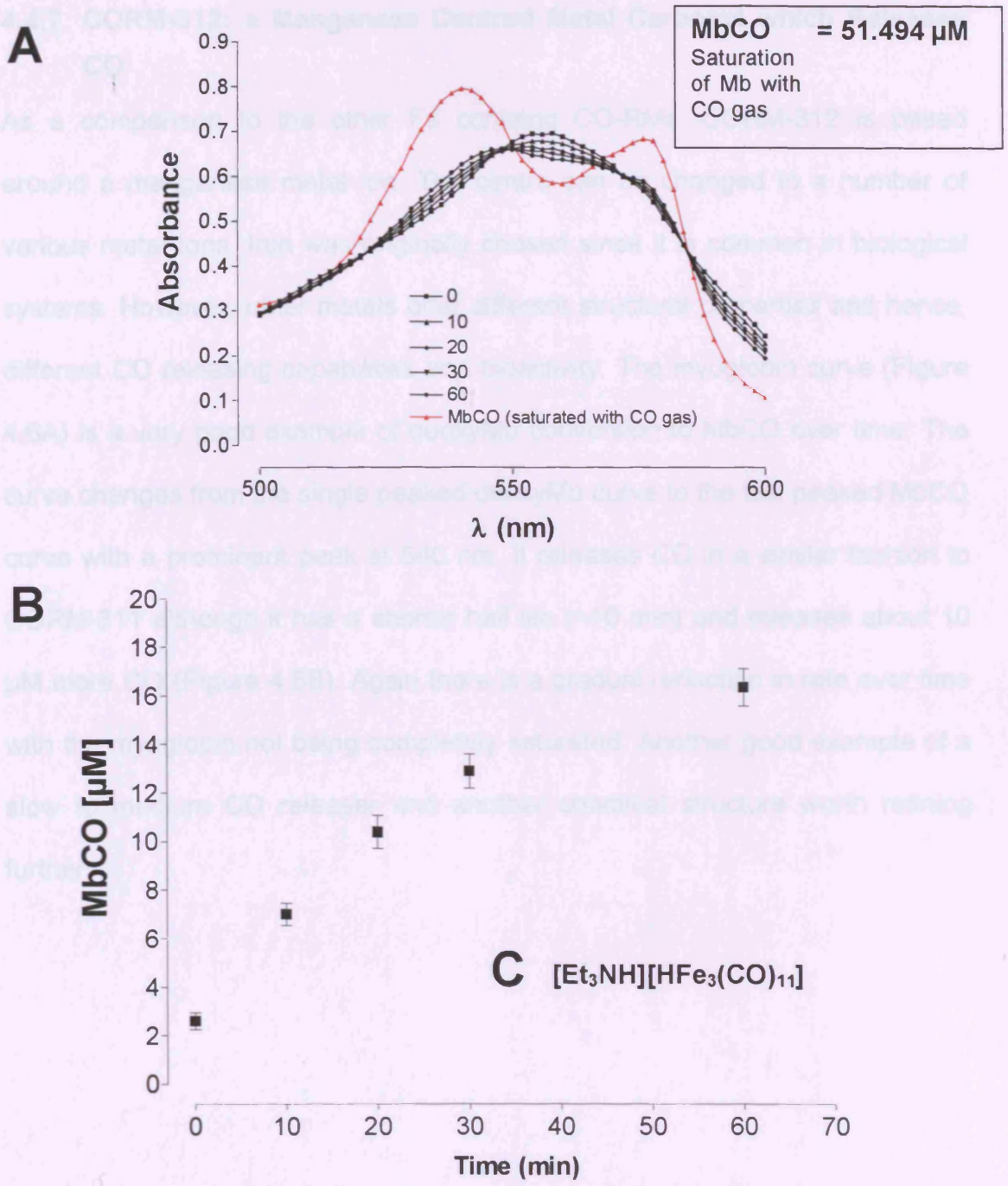


Figure 4.5:- CORM-311 an example of a slow CO releaser.
(A) CORM-311 (40 µM) was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-311 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution. (C) The chemical formula of CORM-311.

4.4.7 CORM-312: a Manganese Centred Metal Carbonyl which Releases CO

As a comparison to the other Fe containg CO-RMs, CORM-312 is based around a manganese metal ion. The centre can be changed to a number of various metal ions. Iron was originally chosen since it is common in biological systems. However, other metals offer different structural properties and hence, different CO releasing capabilities and bioactivity. The myoglobin curve (Figure 4.6A) is a very good example of deoxyMb conversion to MbCO over time. The curve changes from the single peaked deoxyMb curve to the two peaked MbCO curve with a prominent peak at 540 nm. It releases CO in a similar fashion to CORM-311 although it has a shorter half life (~10 min) and releases about 10 μM more CO (Figure 4.6B). Again there is a gradual reduction in rate over time with the myoglobin not being completely saturated. Another good example of a slow to medium CO releaser and another chemical structure worth refining further.

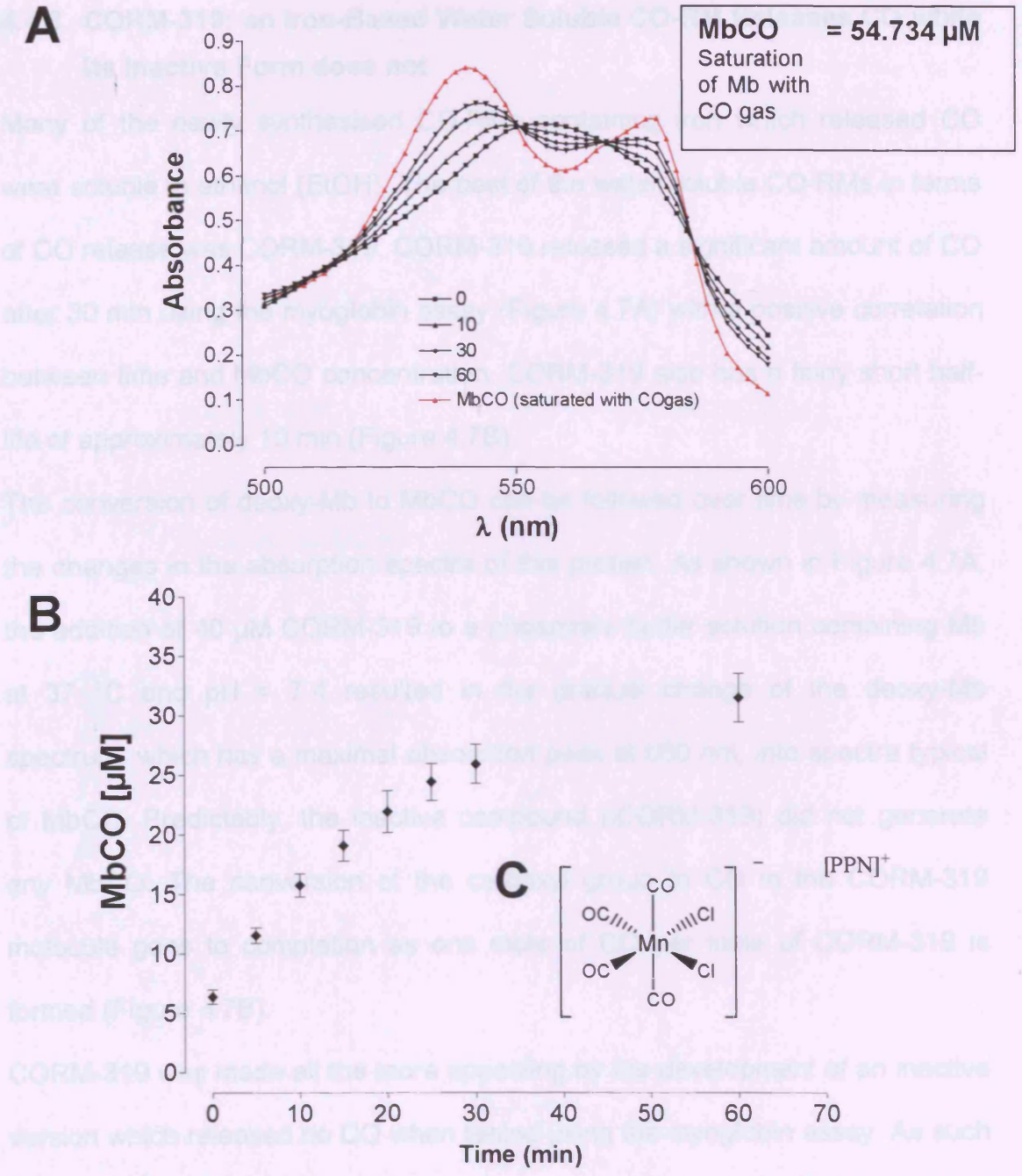


Figure 4.6:- CORM-312: a manganese centred metal carbonyl which releases CO.

(A) CORM-312 (40 µM) was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-312 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution. (C) Chemical structure of CORM-312.

4.4.8 CORM-319: an Iron-Based Water Soluble CO-RM Releases CO while its Inactive Form does not

Many of the newly synthesised CO-RMs containing iron which released CO were soluble in ethanol (EtOH). The best of the water soluble CO-RMs in terms of CO release was CORM-319. CORM-319 released a significant amount of CO after 30 min using the myoglobin assay (Figure 4.7A) with a positive correlation between time and MbCO concentration. CORM-319 also has a fairly short half-life of approximately 10 min (Figure 4.7B).

The conversion of deoxy-Mb to MbCO can be followed over time by measuring the changes in the absorption spectra of this protein. As shown in Figure 4.7A, the addition of 40 μM CORM-319 to a phosphate buffer solution containing Mb at 37 °C and pH = 7.4 resulted in the gradual change of the deoxy-Mb spectrum, which has a maximal absorption peak at 560 nm, into spectra typical of MbCO. Predictably, the inactive compound (iCORM-319) did not generate any MbCO. The conversion of the carboxyl group to CO in the CORM-319 molecule goes to completion as one mole of CO per mole of CORM-319 is formed (Figure 4.7B).

CORM-319 was made all the more appealing by the development of an inactive version which released no CO when tested using the myoglobin assay. As such CORM-319 was deemed a compound of significant interest and its bioactivity would be tested.

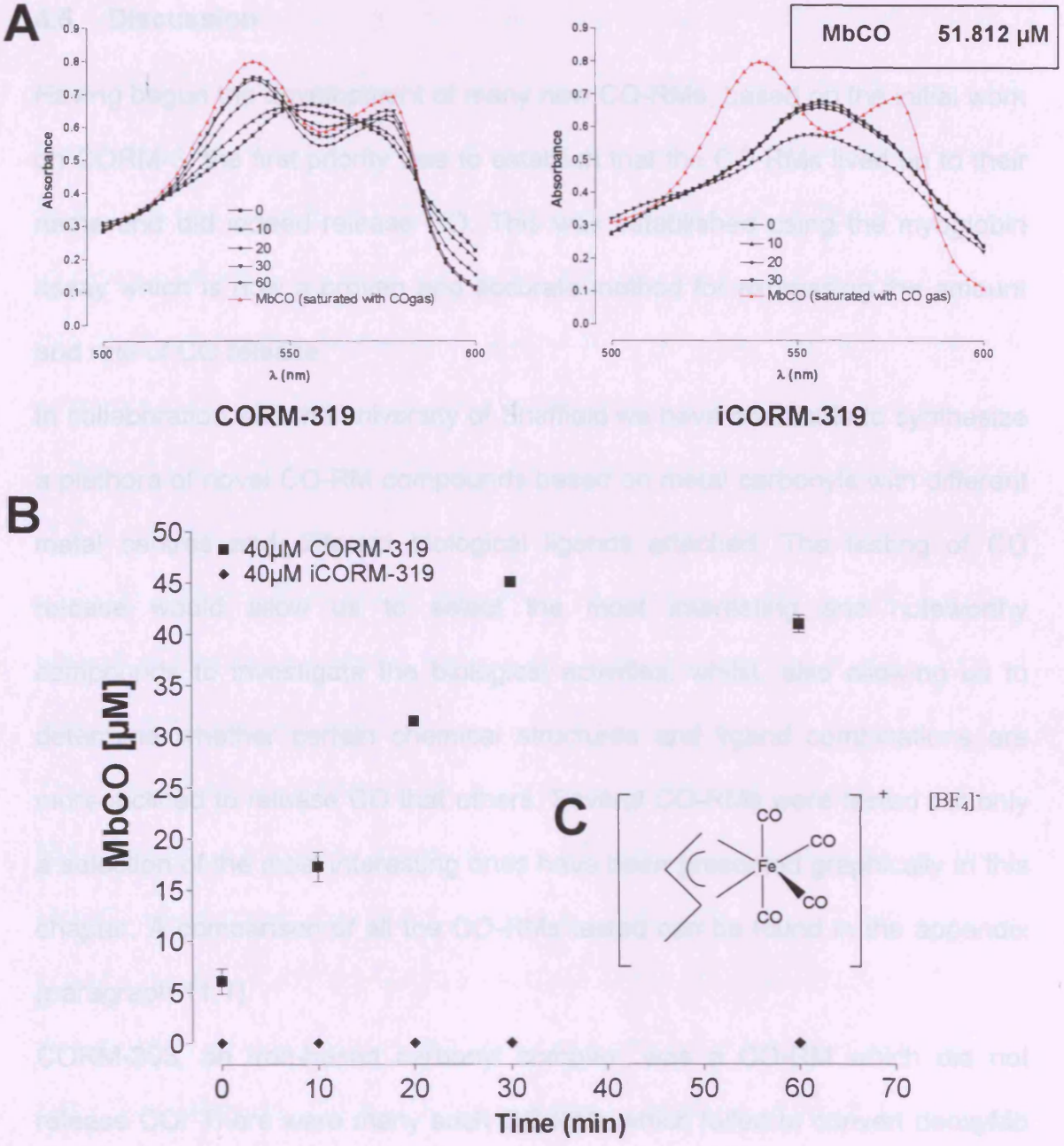


Figure 4.7:- CORM-319: an iron-based water soluble CO-RM releases CO while its inactive form does not.
Prior to testing using the myoglobin assay iCORM-319 was prepared. This was done by heating the stock to 100°C for 10 min followed by the addition of 1 drop of 1 M HCl and finally bubbling with nitrogen to displace any liberated CO from solution. Subsequently (A) iCORM-319 or CORM-319 (40 µM) were added to a 66 µM myoglobin solution. Readings charting any conversion of deoxyMb to MbCO were taken every 5 min for the first 30 min and then once more after an hour. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution. (C) Chemical structure of CORM-319.

4.5 Discussion

Having begun the development of many new CO-RMs, based on the initial work on CORM-3, the first priority was to establish that the CO-RMs lived up to their name and did indeed release CO. This was established using the myoglobin assay which is now a proven and accurate method for expressing the amount and rate of CO release.

In collaboration with the university of Sheffield we have been able to synthesize a plethora of novel CO-RM compounds based on metal carbonyls with different metal centres and different biological ligands attached. The testing of CO release would allow us to select the most interesting and noteworthy compounds to investigate the biological activities, whilst, also allowing us to determine whether certain chemical structures and ligand combinations are more inclined to release CO that others. Several CO-RMs were tested but only a selection of the most interesting ones have been presented graphically in this chapter. A comparison of all the CO-RMs tested can be found in the appendix (paragraph 11.1).

CORM-303, an iron-based carbonyl complex, was a CO-RM which did not release CO. There were many such CO-RMs which failed to convert deoxyMb to MbCO and subsequently did not release any CO. These CO-RMs are not totally useless as they might provide us with forms of negative control in that they are metal carbonyls which do not release CO and have similar structured counterparts which do.

CORM-307 and CORM-308 are both potent CO-releasing agents. Upon addition to myoglobin they release their maximal concentration of CO instantaneously in a 1:1 ratio with compound concentration (40 μM CO-RM = 40

μM MbCO concentration). A useful facet of these two CO-RMs is that inactive versions of each (iCORM-307 and iCORM-308) have been prepared. Compared to their active counterparts, both inactive compounds release almost no CO (20 fold lower for iCORM-307 and 4 fold lower for iCORM-308). As such elucidation of their biological activity will be easier and offer a greater insight into their mechanism of action. It should be noted that both are similar CO releasers to CORM-3, although, they are soluble in ethanol and not water.

CORM-311 and CORM-312 are also soluble in ethanol but offer a slower kinetic of CO release. CORM-311 has a half-life of ~15 min while CORM-312 has a ~10 min half-life. Both would provide a useful tool to study prolonged CO release in biological systems, particularly in an inflammatory or some form of chronic diseased state model.

The final CO-RM evaluated in this chapter was CORM-319, a water soluble CO-RM, capable of a slow kinetic release of ~15 min but delivery of an equivalent MbCO concentration as CORM-307 and CORM-308. Such a powerful CO release combined with the slow kinetics and water solubility makes CORM-319 an attractive and potentially important compound in the long-term study of CO-RMs. The allure of CORM-319 was increased when an inactive version was prepared. CORM-319 is a very promising CO-RM determining its bioactivity is of utmost importance.

The CO-RMs selected to be investigated further after determining the amount and kinetics of CO release would first have to be tested for cellular toxicity, again an attribute that will no doubt be dependent on its chemical structure and the type of ligand coordinated to the metal centre.

4.6 Conclusion

The diverse chemistry of transition metal carbonyls makes them excellent compounds for deriving novel CO releasing molecules from. The ease of synthesis and variation means molecules can be generated that release CO slowly, quickly, in large or small amounts and have varying degrees of solubility in aqueous solutions or are lipid soluble. The further elucidation of these compounds will allow a greater understanding of their action and for the refinement of their properties to generate new and improved versions better suited to specific roles.

5 Chapter 5: Search for new CO-RMs: characterisation of biological features

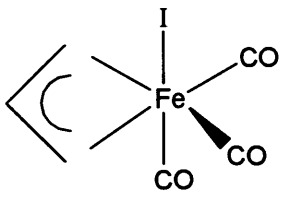
5.1 Introduction

Having established which of the new CO-RMs release CO, the biological activity of each was investigated. Firstly, it was important to identify which, if any, of the CO-RMs was toxic and thus incompatible with the macrophage model used. Although all the CO-RMs contained biological ligands, they also contain metal centres and are completely novel molecules, thus determining toxicity was an important starting point. Any CO-RMs found to be toxic would then be discarded and the remaining ones would be used to investigate various biological activities. The main aim of this was to establish CO as the mediator of action for CO-RMs.

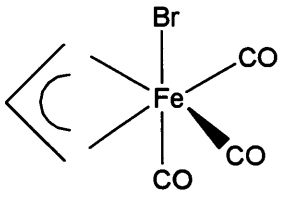
As with CORM-3, for the purpose of this Thesis much of the work centred on the anti-inflammatory action of CO in a murine macrophage model of inflammation triggered using LPS. Further work includes investigating the effect of CO-RM on HO activity, since CO-RMs contain a transition metal, it was reasonable to think that this could activate HO-1 and elicit any of the effects otherwise attributed to CO release. The structures of the CO-RMs tested in this chapter can be seen in Figure 5.1. Note how they all have a similar overall structure and that variations lie in generally only one of the ligands bound to the metal centre.

The findings from both Chapter 2 and this chapter detailing the overall effects of the new CO-RMs could then be used by our chemist collaborator (Professor Brian Mann) to develop less toxic, more water soluble and better CO-releasing

compounds. As such, the investigation of these molecules is an ongoing process to derive the best combination of factors.



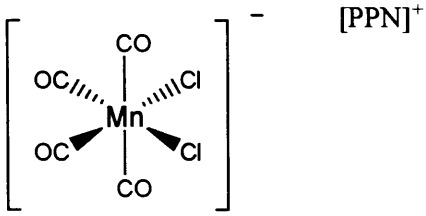
CORM-307



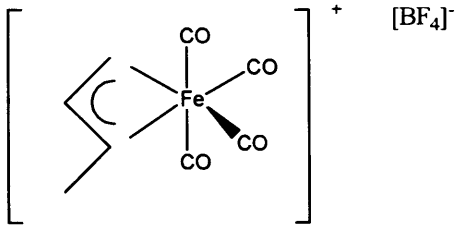
CORM-308

$[Et_3NH][HFe_3(CO)11]$

CORM-311



CORM-312



CORM-319

Figure 5.1:- Structure of CO-RMs to be tested in this Chapter.
Note the underlying similarity in structure.

5.2 Objective

Test compounds deemed to be worthy of further study from Chapter 4 to determine their toxicity, effect on the HO system and regulation of the inflammatory response.

5.3 Experimental Protocol

The following section details the protocol of specific experiments of which more precise methods can be found in the Material and Methods chapter. Stock solutions of CO-RMs (10 mM) were freshly prepared before each experiment. CO-RMs was layered with N_2 gas prior to returning to storage in the freezer at -20°C.

5.3.1 Chemicals and reagents

CORM-307, CORM-308, CORM-311, CORM-312 and CORM-319 were synthesised by Professor Brian Mann (University of Sheffield). CORM-307, CORM-308, CORM-311 and CORM-312 were freshly prepared as 10 mM stock solutions in ethanol. While CORM-319, was prepared as a 10 mM stock solution in pure distilled water. CORM-319 was inactivated (iCORM-319) by adding it to complete DMEM and leaving for 18 h at 37 °C in a 5% CO_2 humidified atmosphere to liberate CO after which the solution was bubbled with nitrogen (N_2) gas for 5 min to remove any CO trapped in solution. Both CORM-307 and CORM-308 could be inactivated (iCORM-307 and iCORM-308) by leaving them in ethanol for 48 h to release their CO (as determined by the myoglobin assay). Lipopolysaccharide (LPS - *E.Coli* serotype 026:B6) was obtained from Sigma. All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

5.3.2 Myoglobin Assay

To prepare a stock of CO-RM for the myoglobin assay, 1 mg of CO-RM was added to 100 µl of dH_2O to give a main stock. From this 2, 4, 8 and 12 mM

working stocks were prepared. The myoglobin was warmed (37 °C), sodium dithionite (0.1 %) added and a 1 ml sample of the myoglobin measured for the deoxy-Mb curve. This 1 ml sample of deoxy-Mb was then bubbled with CO gas for 10 sec to saturate the myoglobin and give a MbCO curve. Six cuvettes were then loaded into the spectrophotometer carousel with 1 ml of deoxy-Mb in each, 5 µl of 2, 4, 8, and 12 mM CO-RM working stocks were then added in duplicate to each cuvette. The resultant final concentrations of CO-RM being 10 (from 2 mM), 20 (from 4 mM), 40 (from 8 mM) and 60 µM (from 12 mM). The myoglobin was then mixed gently using a pipette and layered with 500 µl of mineral oil. The samples were then read at set intervals for a set period of time. CORM-319 stability was determined by preparing a stock solution of CORM-319 in pure distilled water. From this stock 60 µM CORM-319 was added to myoglobin and left to release CO for 10 min at 37 °C after which the spectra was read. This was repeated at 5 min intervals using the original stock.

5.3.3 Cell Culture

Murine RAW264.7 monocyte macrophages and Girardi (human heart) cells were cultured as per Material and Methods. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

5.3.4 Experimental Protocol

To give an indication of cell viability macrophages were treated with CORM-307, CORM-308, CORM-311, CORM-312 (10, 50 and 100 µM) and CORM-319 (10, 50, 100, 500, 750 and 1000 µM) for 24 h after which an Alamar blue assay

was run. CORM-319 (10, 50, 100, 500, 750 and 1000 μM) toxicity was also determined by LDH and Trypan blue exclusion after 24 h incubation of macrophages. CO-RMs of which iCO-RMs had been generated (CORM-307, CORM-308 and CORM-319) were also tested in the same manner. The non-toxic CO-RMs were tested for their anti-inflammatory effect on nitrite production. Macrophages were exposed for 24 h to LPS (1 μg/ml) in the presence or absence of CORM-311 or CORM-319 (10, 50 and 100 μM) and nitrite levels were determined at the end of the incubation. Experiments were repeated with the negative control iCORM-319 to assess whether the effects observed were due to the CO liberated by the CO-RM or caused by other components of the molecule. The effects of CORM-311, CORM-319 and iCORM-319 on the haem oxygenase pathway were also investigated. Specifically, murine macrophages and Girardi cells were treated for 6 h in the presence of 10, 50 and 100 μM CO-RM after which haem oxygenase activity was determined.

5.3.5 Assay for Nitrite Levels

Nitrite levels were determined using the Griess method. The measurement of this parameter is widely accepted as indicative of NO production. Briefly, the medium from treated cells cultured in 24 well plates was removed and placed into a 96 well plate (50 μl per well). The Griess reagent was added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0 μM to 300 μM in cell culture medium).

5.3.6 Assay for Haem Oxygenase Activity

Haem oxygenase activity was determined in RAW264.7 cells and Girardi cells after various treatments. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH = 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 μM). The reaction was conducted at 37 °C in the dark for 1 h and terminated by the addition of 1 ml chloroform; the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

5.3.7 Cell Viability

Alamar Blue

Cell metabolism was determined using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK). The assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised (blue) form to a reduced (red) form. The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control.

Trypan Blue

The status of the cell membrane was investigated using Trypan blue. The medium of treated cells is removed and the cells then washed with PBS 10X.

Then a 1:1 solution of PBS (10X) and Trypan blue dye was added to cells. The dye permeates cell in which the membrane has been compromised. By counting the number of cell dyed compared to those which are not the number of viable cells can be determined.

Lactate Dehydrogenase

Damage of the phopholipid bi-layer of cells was investigated using an LDH assay kit and carried out according to the manufacturer's instructions (Roche, UK). The medium of treated cells is collected, spun to remove any cellular debris and then a reaction mixture containg an INT dye added. LDH activity reflects its release from the cell membrane and a loss of viability.

5.3.8 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$.

5.4 Results

5.4.1 Determining the viability of CO-RMs

Having established in Chapter 4 a number of CO-RMs deemed worthy for further study the first requirement was to test their toxicity in murine macrophages (the cells used in the LPS model of nitrite generation). In some cases we were also able to test iCO-RM versions; however, not all the CO-RMs tested could be inactivated owing to their chemistry. It was decided for efficiencies sake to test the CO-RMs using the Alamar blue assay which is quick and simple to perform and is widely used as an indicator of cell viability (Benghuzzi 1995; Nakayama et al. 1997; Voytik-Harbin et al. 1998). Both CORM-307 and CORM-308 displayed very similar effects on cell health (Figure 5.2 and Figure 5.3). Toxicity was evident at only 10 μM CORM-307 and CORM-308, while at 50 μM there was a 100 % loss in viability using both CO-RMs (Figure 5.2A and Figure 5.3A). In both cases iCO-RM version of each displayed an almost identical effect on cell viability as the active counterpart (Figure 5.2B and Figure 5.3B). CORM-311 meanwhile, had no effect on cellular metabolism at any concentration thus, ruling out an effect of the methanol used to solubilise itself, CORM-307, CORM-308 and CORM-312 (Figure 5.4A). In a similar fashion to both CORM-307 and CORM-308, CORM-312 was almost 95 % toxic at 50 μM but unlike the others was only 5 % toxic at 10 μM (Figure 5.4B). Finally, CORM-319, a charged variation on the other CO-RMs rendering it water soluble, displayed little toxicity up to 500 μM. There was a 20 % reduction in cell viability at 750 μM and an almost 90 % reduction at 1000 μM (Figure 5.5A). But importantly, no effects at the concentrations regularly used. These results were almost mirrored by those of iCORM-319 in which 1000 μM iCORM-319 was

slightly less toxic than the active version (Figure 5.5B). Based on these data CORM-311 and CORM-319 were taken forward for further study. CORM-319 especially interesting due to its water solubility and presence of an iron.

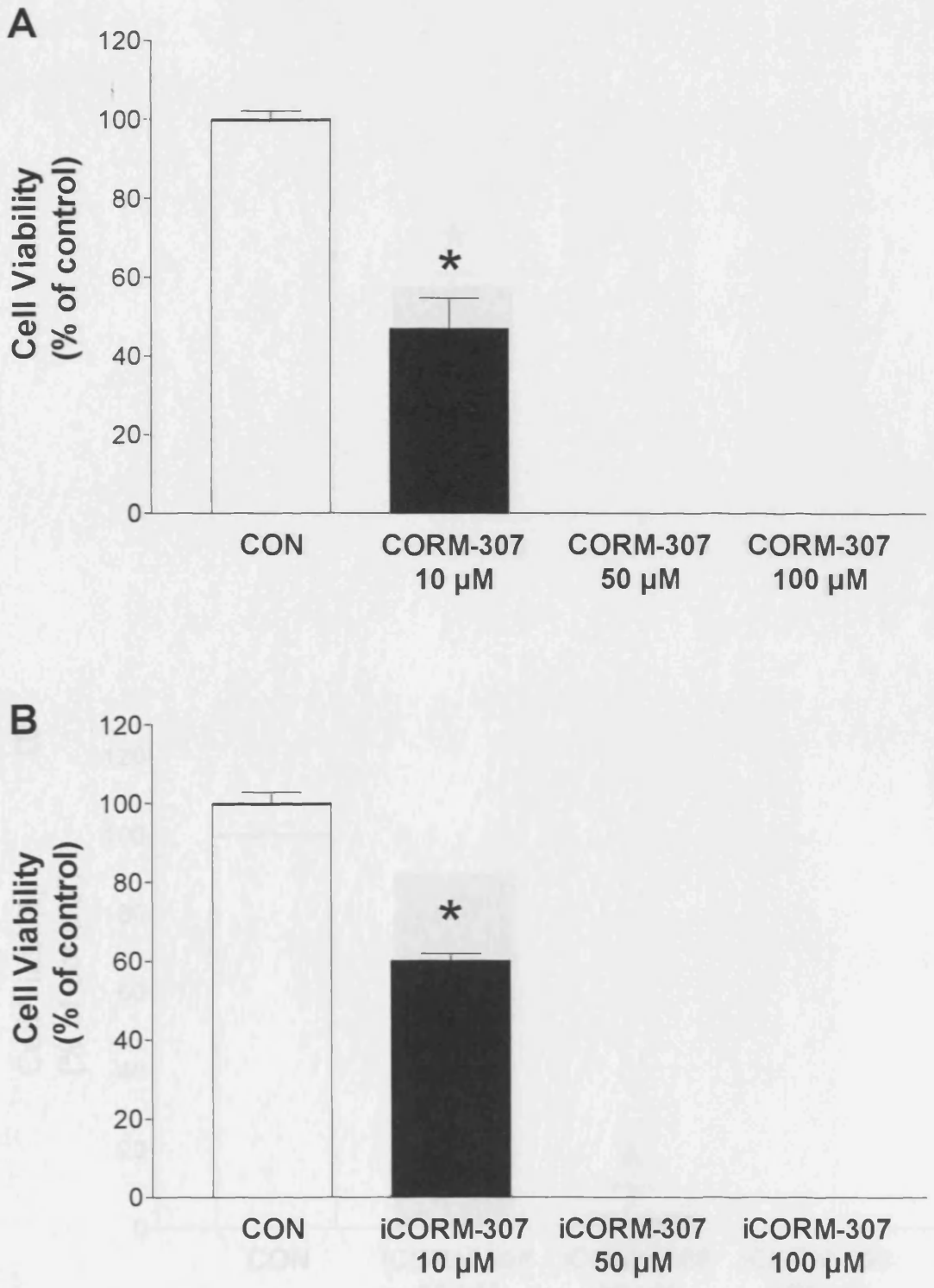


Figure 5.2:- Effect of CORM-307 and iCORM-307 on cellular metabolism. Cell metabolism was assessed 24 h after exposure of macrophages to (A) CORM-307 (10-100 µM) or (B) iCORM-307 (10-100 µM) using the Alamar blue viability assay. Viability was expressed as a percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

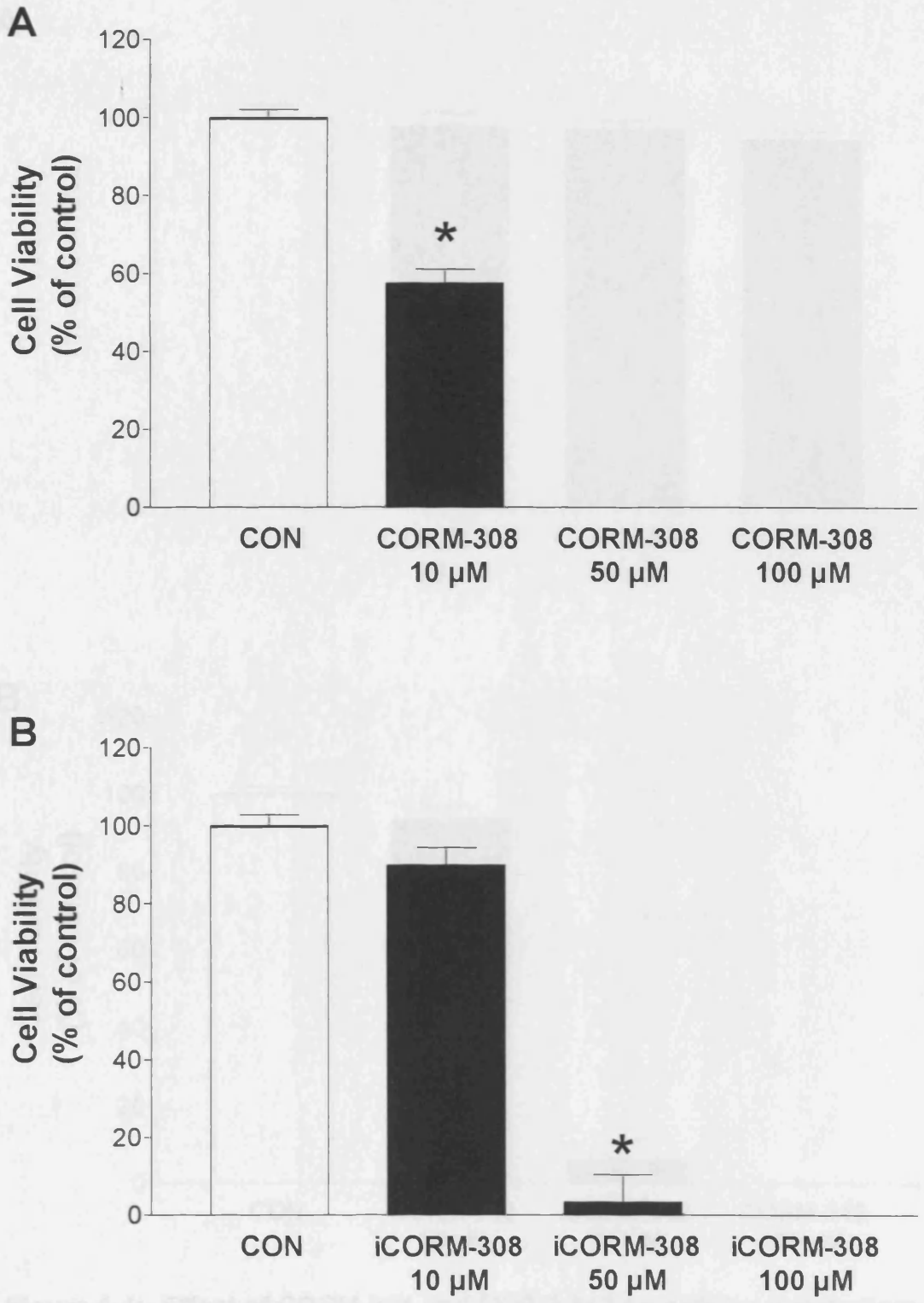


Figure 5.3:- Effect of CORM-308 and iCORM-308 on cellular metabolism. Cell metabolism was assessed 24 h after exposure of macrophages to (A) CORM-308 (10-100 µM) or (B) iCORM-308 (10-100 µM) using the Alamar blue viability assay. Viability was expressed as a percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

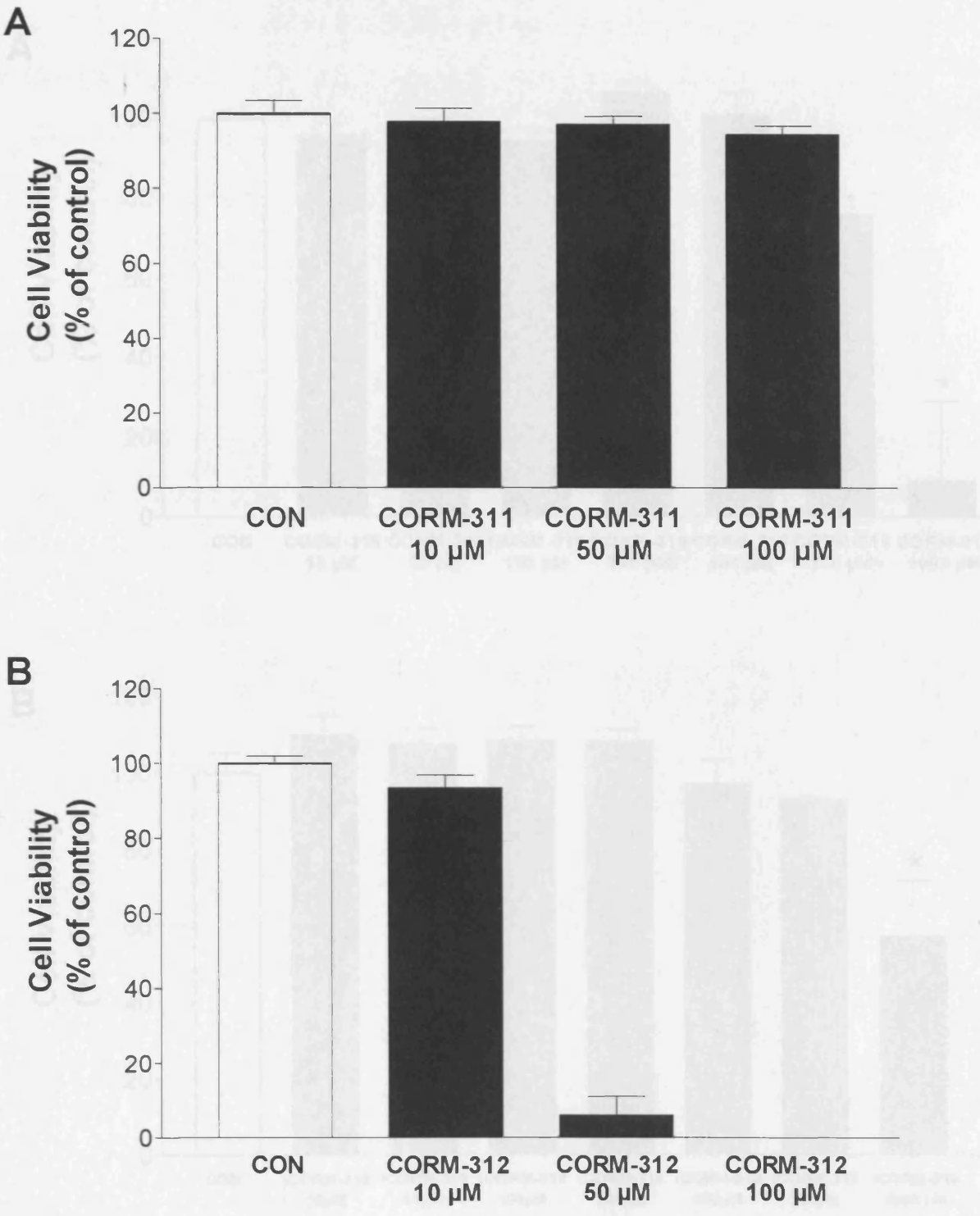


Figure 5.4:- Effect of CORM-311 and CORM-312 on cellular metabolism. Cell metabolism was assessed 24 h after exposure of macrophages to (A) CORM-311 (10-100 µM) or (B) CORM-312 (10-100 µM) using the Alamar blue viability assay. Viability was expressed as a percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

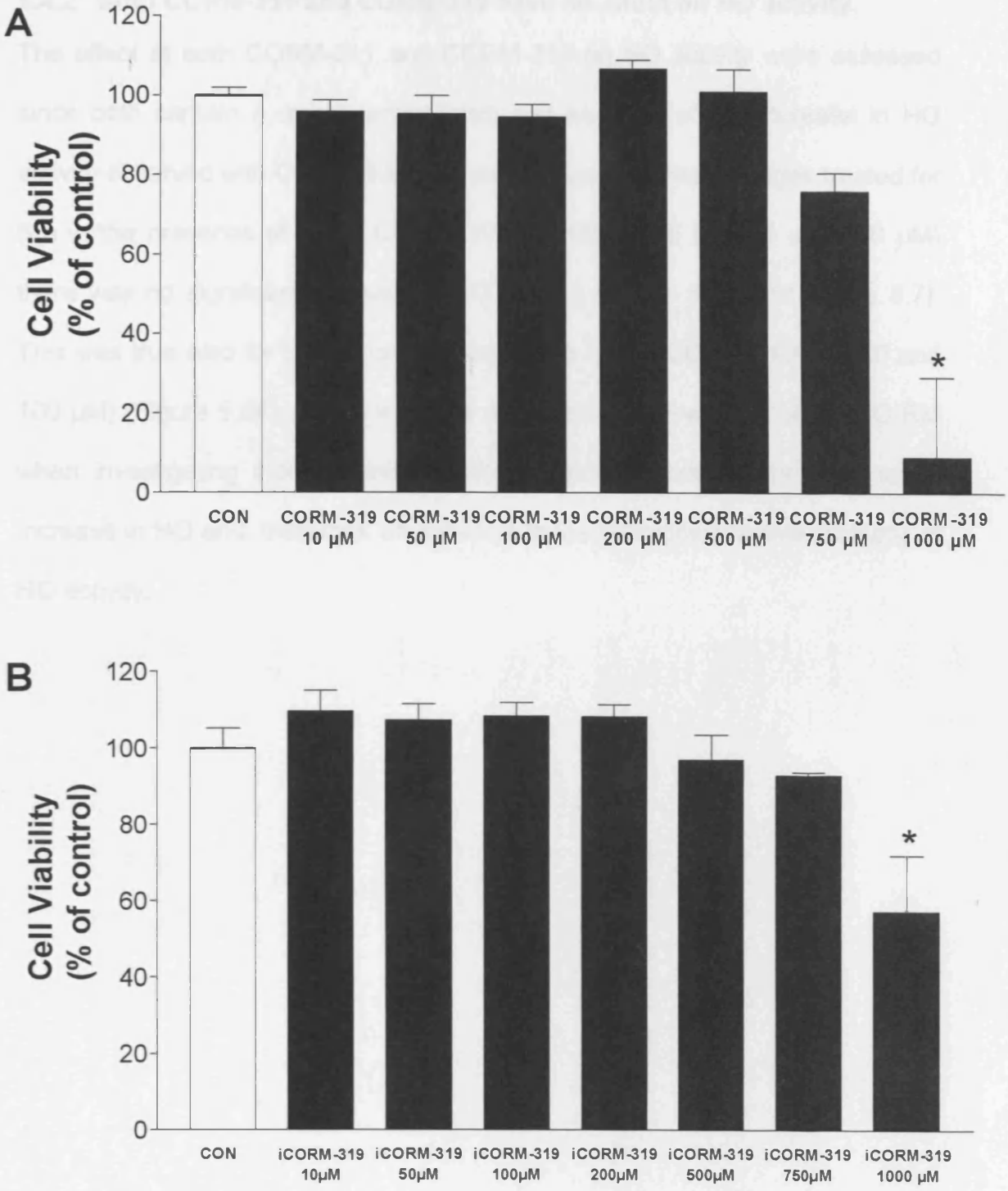


Figure 5.5:- Effect of CORM-319 and iCORM-319 on cellular metabolism. Cell metabolism was assessed 24 h after exposure of macrophages to (A) CORM-319 (10-1000 µM) or (B) iCORM-319 (10-1000 µM) using the Alamar blue viability assay. Viability was expressed as a percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

5.4.2 Both CORM-311 and CORM-319 have no effect on HO activity.

The effect of both CORM-311 and CORM-319 on HO activity were assessed since both contain a metal centre (iron) and because of the increase in HO activity observed with CORM-3 in Chapter 3. In murine macrophages treated for 6 h in the presence of either CORM-311 or CORM-319 (10, 50 and 100 μM) there was no significant increase in HO activity (Figure 5.6B and Figure 5.7). This was true also for Girardi cells treated for 6 h with CORM-319 (10, 50 and 100 μM) (Figure 5.6A). In this instance any effect observed with either CO-RM when investigating their anti-inflammatory action can not be attributed to an increase in HO and, therefore, an increase in the biologically active products of HO activity.

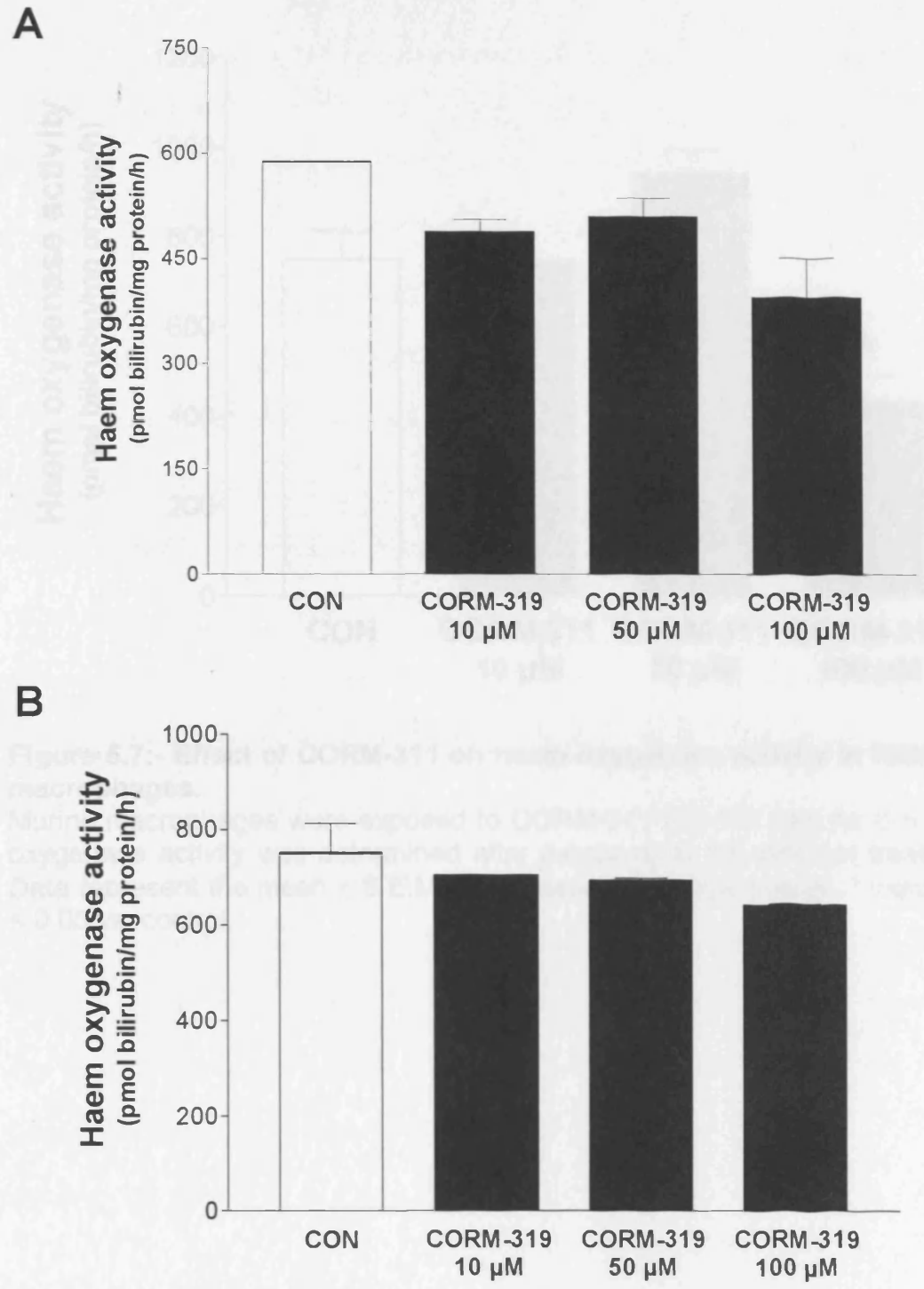


Figure 5.6:- Effect of CORM-319 on haem oxygenase activity in various cell lines.

(A) Girardi cells were treated with CORM-319 (10-100 µM) for 6 h. (B) RAW264.7 macrophages were exposed to CORM-319 (10-100 µM) for 6 h. Haem oxygenase activity was determined after exposure to the different treatments. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

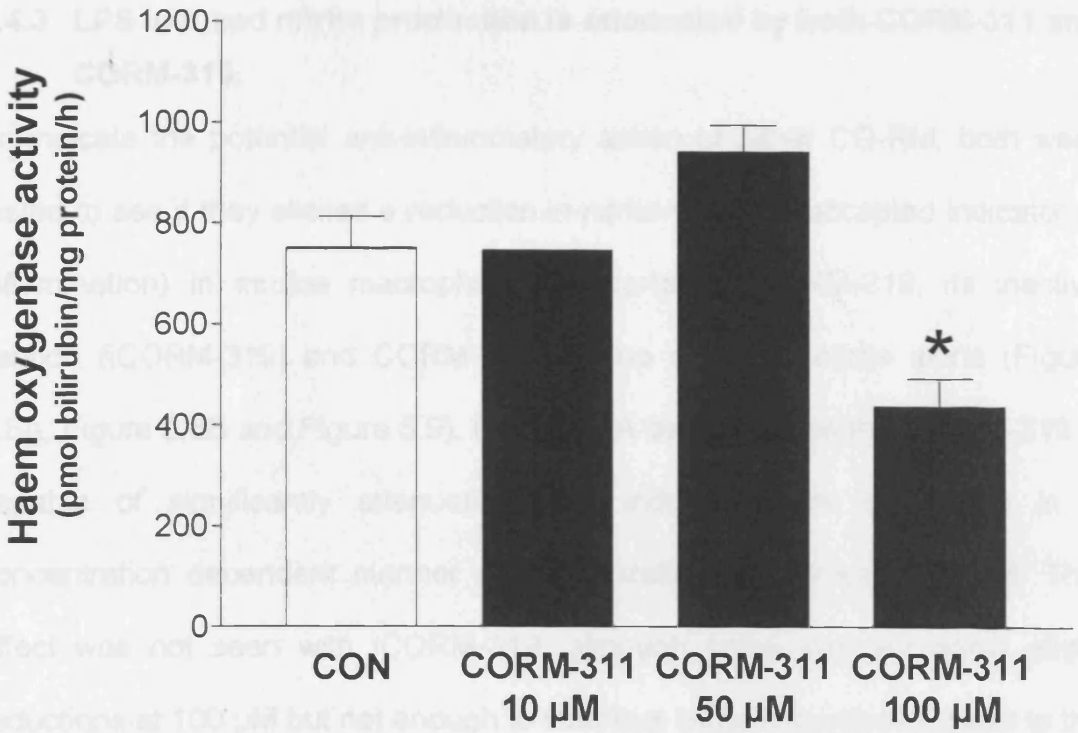


Figure 5.7:- Effect of CORM-311 on haem oxygenase activity in RAW264.7 macrophages.
Murine macrophages were exposed to CORM-311 (10-100 µM) for 6 h. Haem oxygenase activity was determined after exposure to the different treatments. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

5.4.3 LPS induced nitrite production is attenuated by both CORM-311 and CORM-319.

To indicate the potential anti-inflammatory action of either CO-RM, both were tested to see if they elicited a reduction in nitrite (a widely accepted indicator of inflammation) in murine macrophages. Importantly, CORM-319, its inactive version (iCORM-319) and CORM-311 had no effect on nitrite alone (Figure 5.8A, Figure 5.8B and Figure 5.9). Figure 5.8A demonstrates that CORM-319 is capable of significantly attenuating LPS induced nitrite production in a concentration dependent manner at concentrations of 50 and 100 μM. This effect was not seen with iCORM-319, although there was still some slight reductions at 100 μM but not enough to infer that nitrite reduction is linked to the compound itself and not the CO released (Figure 5.8B). CORM-311 meanwhile, is potent at reducing the increased nitrite levels associated with LPS treatment. Indeed, 100 μM CORM-311 reduced nitrite (compared to LPS alone) by around 85 % (Figure 5.9). Since no inactive version of CORM-311 exists the exact mechanism by which CORM-311 works is difficult to speculate on, although, in the case of both CORM-3 and CORM-319 which attenuate LPS induced nitrite generation there is no effect by either inactive version suggesting CO is the mediator of action.

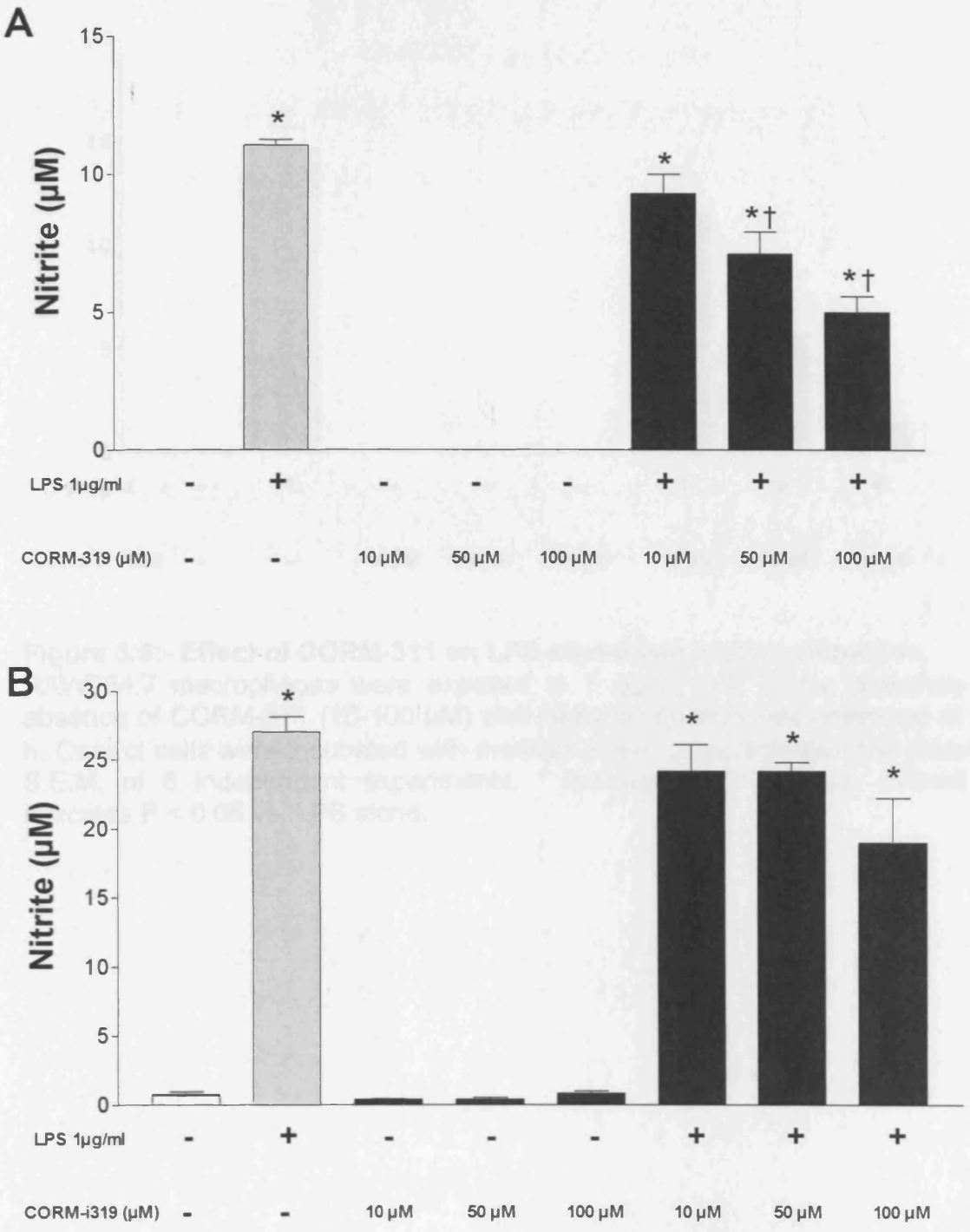


Figure 5.8:- Effect of CORM-319 on LPS-stimulated nitrite production.
(A) RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-319 (10-100 μM) and nitrite production was assessed at 24 h. (B) The inactive compound iCORM-319 (10 - 100 μM) was also used to determine the contribution of CO released by CORM-319 to the observed effect. Control cells were incubated with medium alone. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control; † indicates $P < 0.05$ vs. LPS alone.

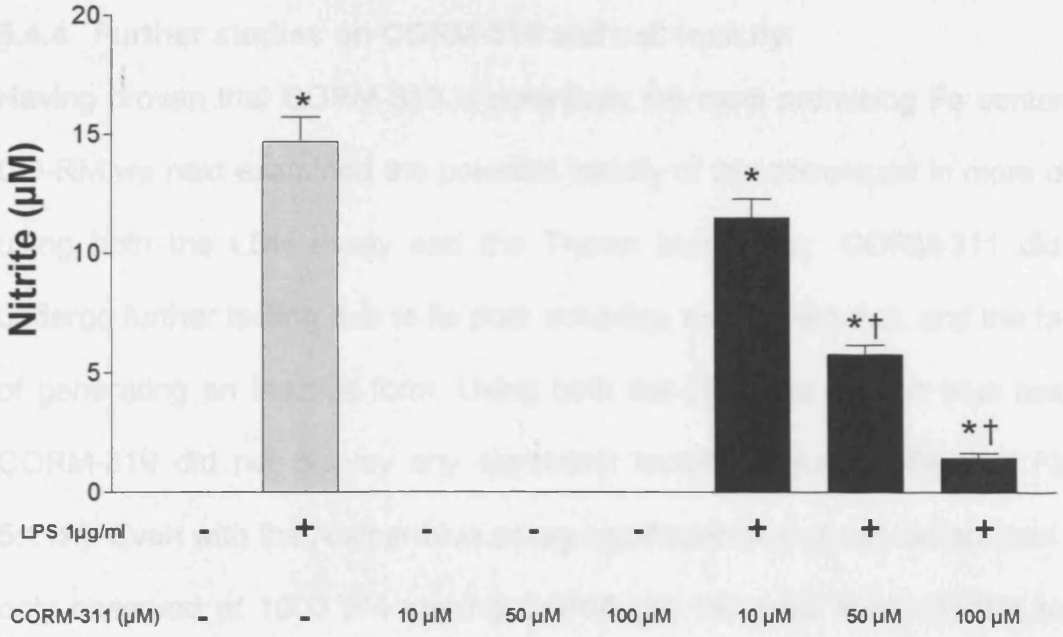


Figure 5.9:- Effect of CORM-311 on LPS-stimulated nitrite production. RAW264.7 macrophages were exposed to 1 µg/ml LPS in the presence or absence of CORM-311 (10-100 µM) and nitrite production was assessed at 24 h. Control cells were incubated with medium alone. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control; † indicates $P < 0.05$ vs. LPS alone.

5.4.4 Further studies on CORM-319 and cell toxicity.

Having proven that CORM-319 is potentially the most promising Fe containing CO-RM we next examined the potential toxicity of this compound in more detail using both the LDH assay and the Trypan blue assay. CORM-311 did not undergo further testing due to its poor solubility, even in ethanol, and the failure of generating an inactive form. Using both the LDH and Trypan blue assays, CORM-319 did not display any significant toxicity (Figure 5.10A and Figure 5.11A). Even with the Alamar blue assay significant loss of cell metabolism was only observed at 1000 μM making CORM-319 the least toxic CO-RM tested thus far. However, the inactive version, iCORM-319, proved to be toxic at concentrations of 500 μM and above in both LDH and Trypan blue assays (Figure 5.10B and Figure 5.11B). LDH measurements showed approximately 50 % cytotoxicity at 1000 μM while the Trypan blue assay suggested 100 % of the cells were dead at this concentration. Both, although not in perfect agreement, are in stark contrast to Alamar blue data which reports only a 40 % drop in cell viability at 1000 μM iCORM-319 (Figure 5.5B). This again highlights the requirement for multiple assays for cell viability. We can conclude from this set of experiments that both CORM-319 and iCORM-319 are not toxic in murine macrophages at the concentrations used in the various treatments but inactivation of CORM-319 leads to a compound capable, at concentrations of 500 μM and above, of interfering with the metabolism of cells and causing damage to the cell membrane.

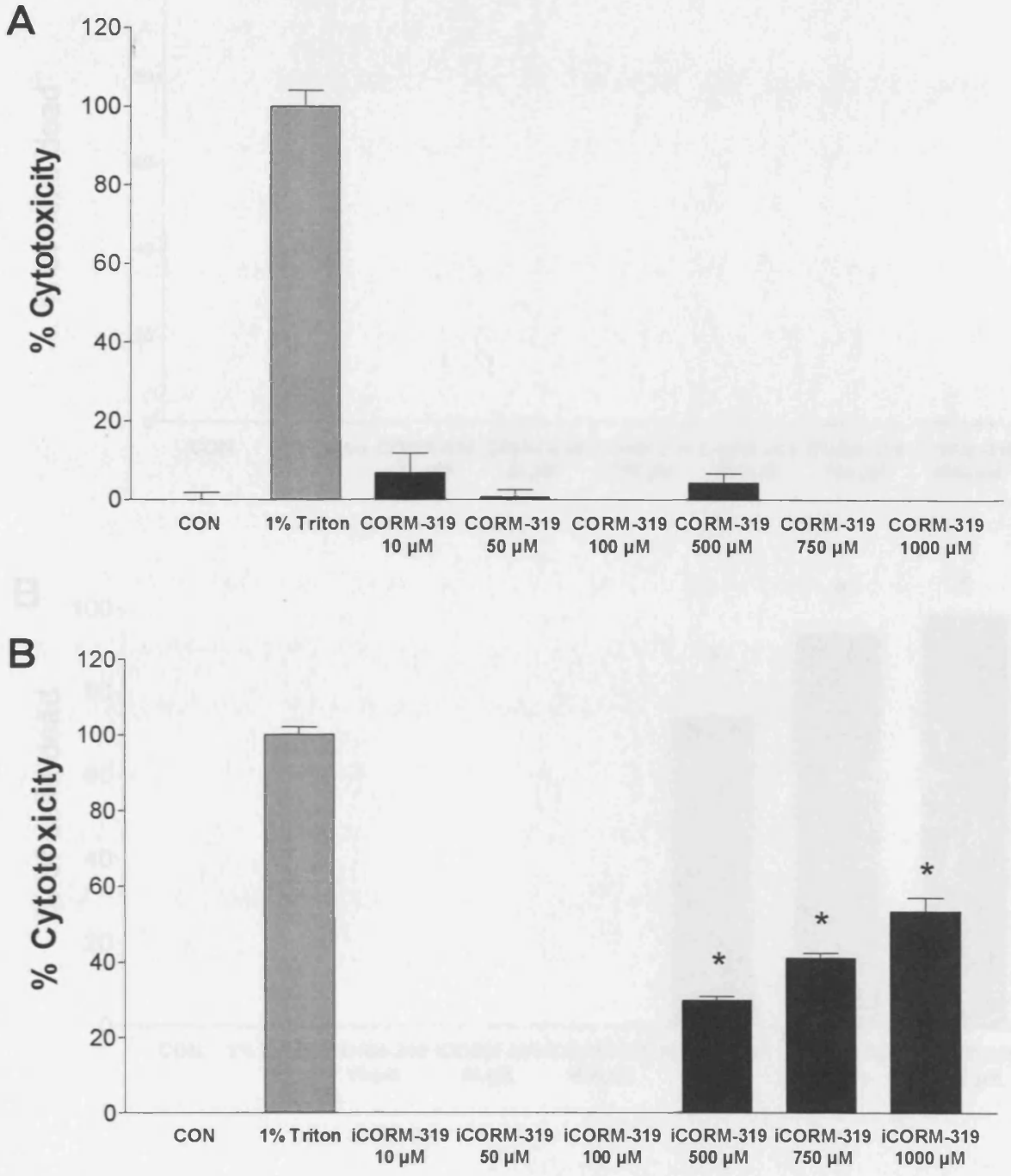


Figure 5.10:- Effect of CORM-319 on LDH release in RAW264.7 macrophages.

The release of LDH was assessed 24 h after exposure of macrophages to (A) CORM-319 (10-1000 µM) or (B) iCORM-319 (10-1000 µM) using an LDH kit purchased from Roche Diagnostics. Viability was expressed as a percentage of maximal damage (triton) and minimal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.005$ vs. control.

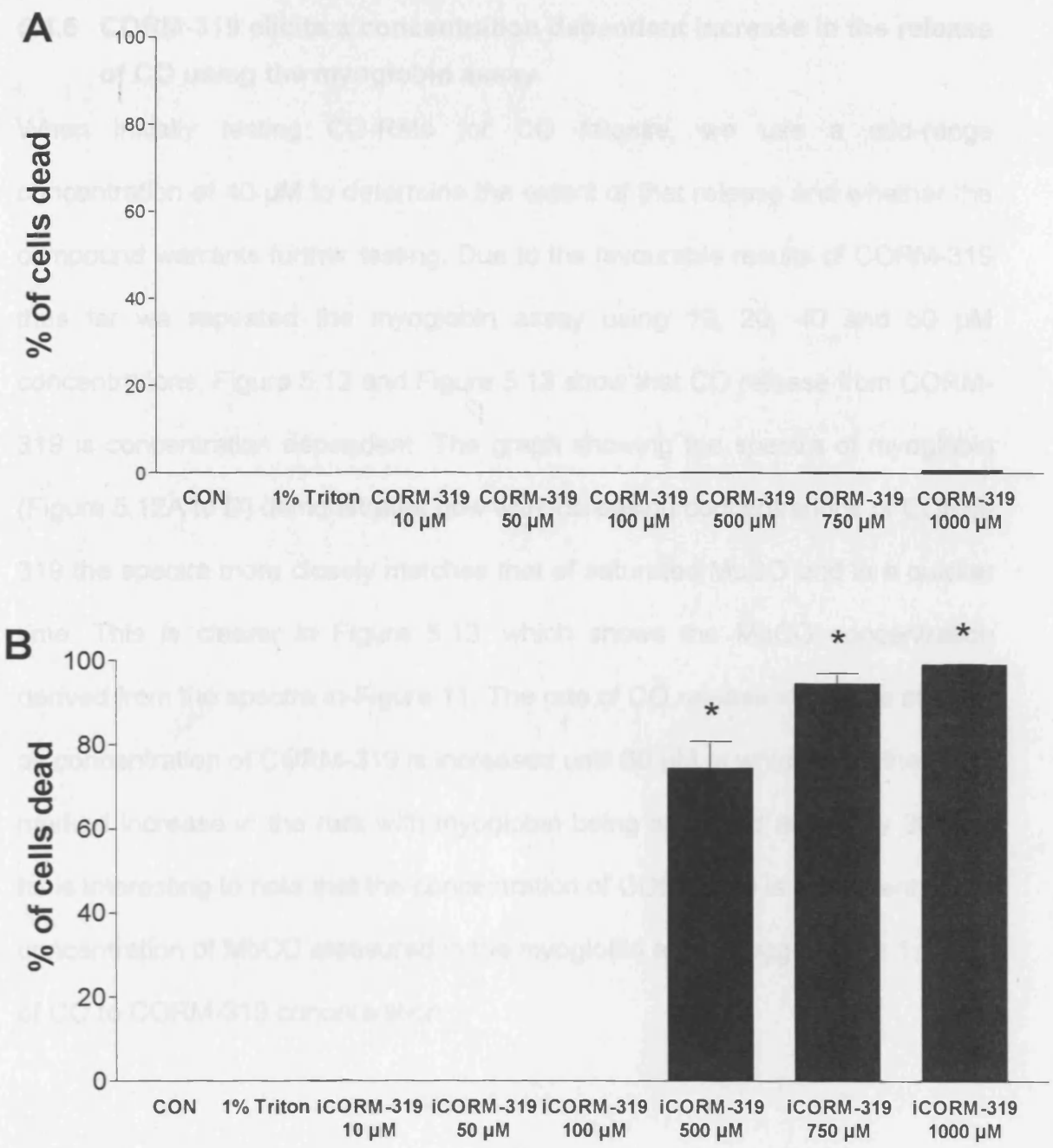


Figure 5.11:- Effect of CORM-319 on cell membrane permeability to Trypan blue dye.

Cell membrane permeability was assessed after 24 h exposure of macrophages to (A) CORM-319 (10-1000 µM) or (B) iCORM-319 (10-1000 µM) using the Trypan blue exclusion assay. Viability was expressed as a percentage of cells stained with the dye compared to the total number of cells. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

5.4.5 CORM-319 elicits a concentration dependent increase in the release of CO using the myoglobin assay.

When initially testing CO-RMs for CO release, we use a mid-range concentration of 40 μM to determine the extent of that release and whether the compound warrants further testing. Due to the favourable results of CORM-319 thus far we repeated the myoglobin assay using 10, 20, 40 and 60 μM concentrations. Figure 5.12 and Figure 5.13 show that CO release from CORM-319 is concentration dependent. The graph showing the spectra of myoglobin (Figure 5.12A to D) demonstrates how with increasing concentrations of CORM-319 the spectra more closely matches that of saturated MbCO and in a quicker time. This is clearer in Figure 5.13, which shows the MbCO concentration derived from the spectra in Figure 11. The rate of CO release increases steadily as concentration of CORM-319 is increased until 60 μM at which point there is a marked increase in the rate with myoglobin being saturated after only 30 min. Its is interesting to note that the concentration of CORM-319 is equivalent to the concentration of MbCO measured in the myoglobin assay suggesting a 1:1 ratio of CO to CORM-319 concentration.

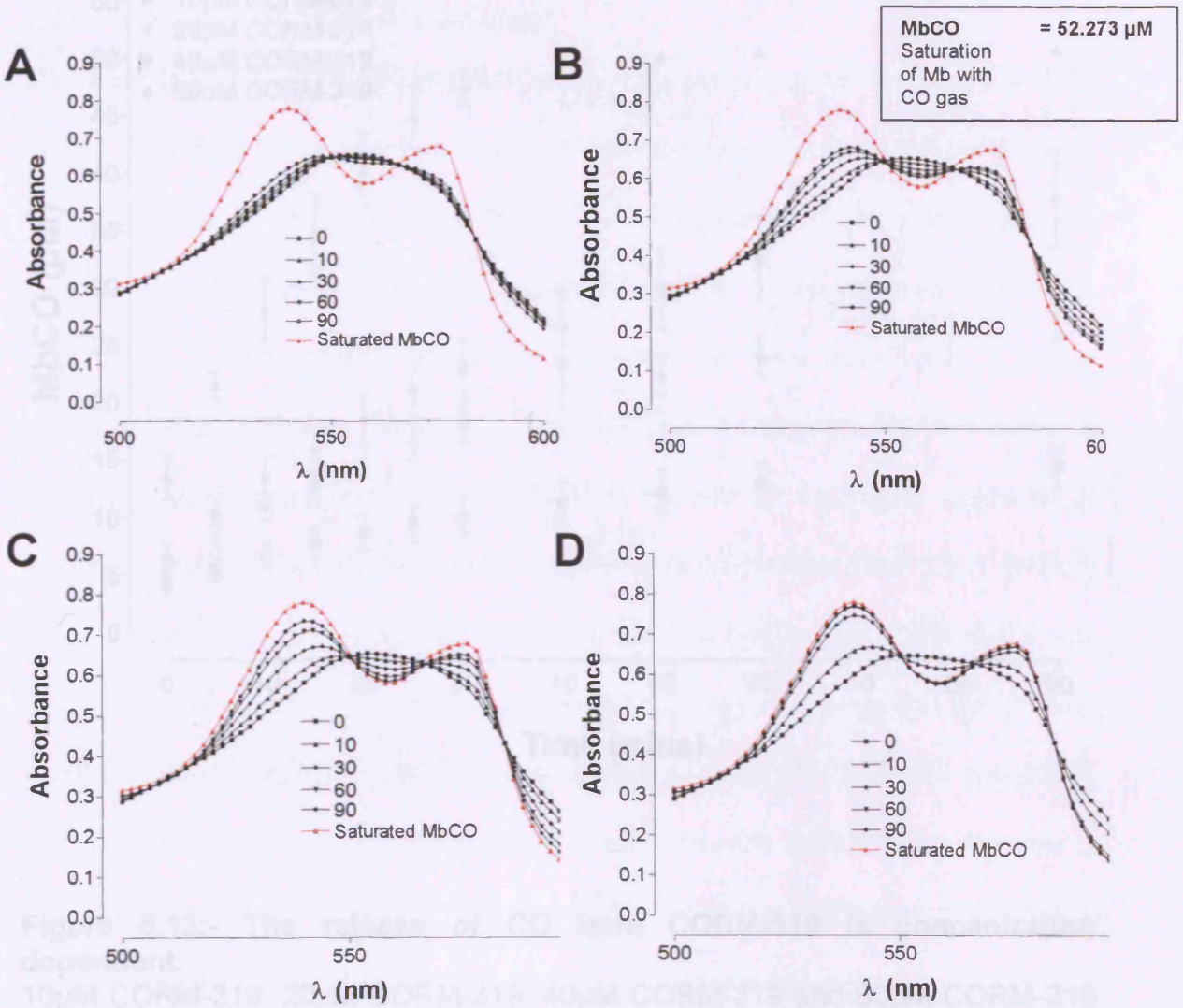


Figure 5.12:- Conversion of deoxy-myoglobin to carboxy-myoglobin by CORM-319.

(A) 10µM CORM-319, (B) 20µM CORM-319, (C) 40µM CORM-319 and (D) 60µM CORM-319 was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CO-RM to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min.

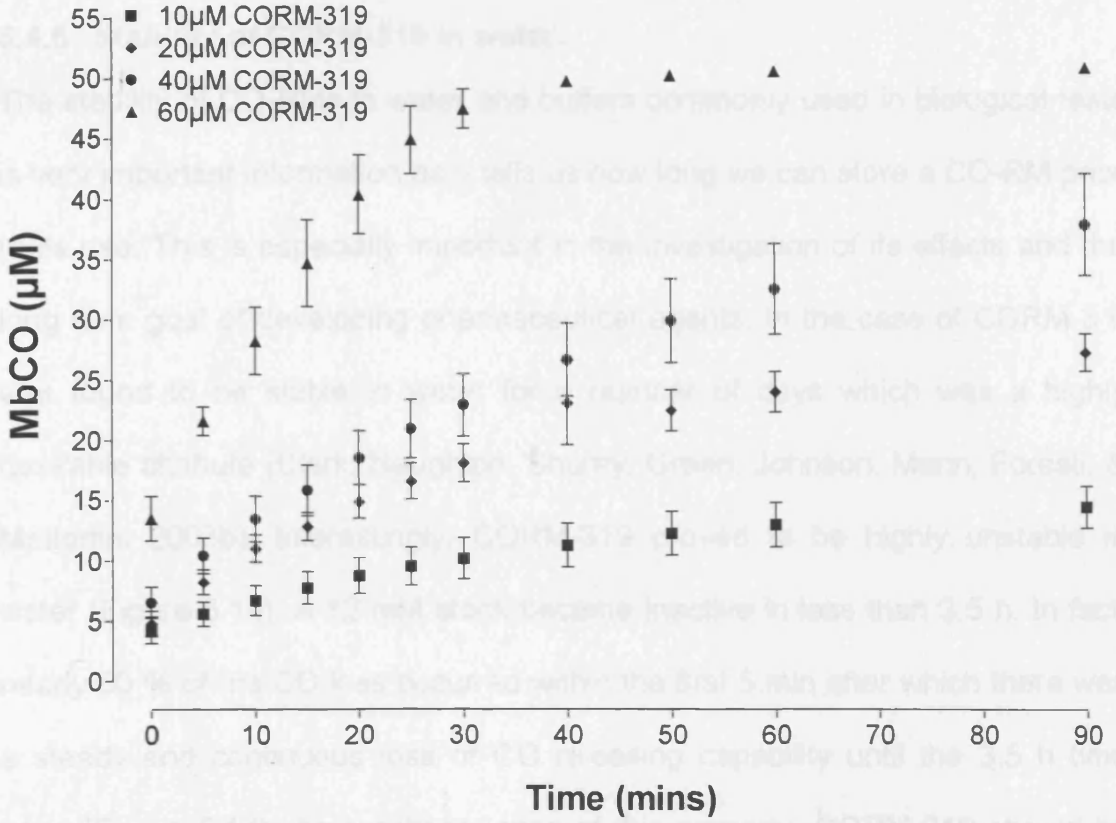


Figure 5.13:- The release of CO from CORM-319 is concentration dependent.
10μM CORM-319, 20μM CORM-319, 40μM CORM-319 and 60μM CORM-319 was added to a 66 μM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CO-RM to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. The MbCO concentrations were derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

5.4.6 Stability of CORM-319 in water.

The stability of CO-RMs in water and buffers commonly used in biological tests is very important information as it tells us how long we can store a CO-RM prior to its use. This is especially important in the investigation of its effects and the long term goal of developing pharmaceutical agents. In the case of CORM-3 it was found to be stable in water for a number of days which was a highly desirable attribute (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). Interestingly, CORM-319 proved to be highly unstable in water (Figure 5.14). A 12 mM stock became inactive in less than 3.5 h. In fact, nearly 50 % of the CO loss occurred within the first 5 min after which there was a steady and continuous loss of CO releasing capability until the 3.5 h time point (Figure 5.14). As a consequence of this property, CORM-319 should be prepared and administered immediately to minimise the spontaneous release of CO associated with preparing the compound in water. However, CORM-319 remained quite stable in air after a number of experiments were conducted over a couple of months using the same vial containing the dry compound.

This set of experiments highlights the need to understand in greater detail the exact chemistry behind the compounds tested. The variation in activities is diverse and only by further exploring the inherent chemical properties of CO-RMs can we fully appreciate the true mechanisms of these compounds in biology.

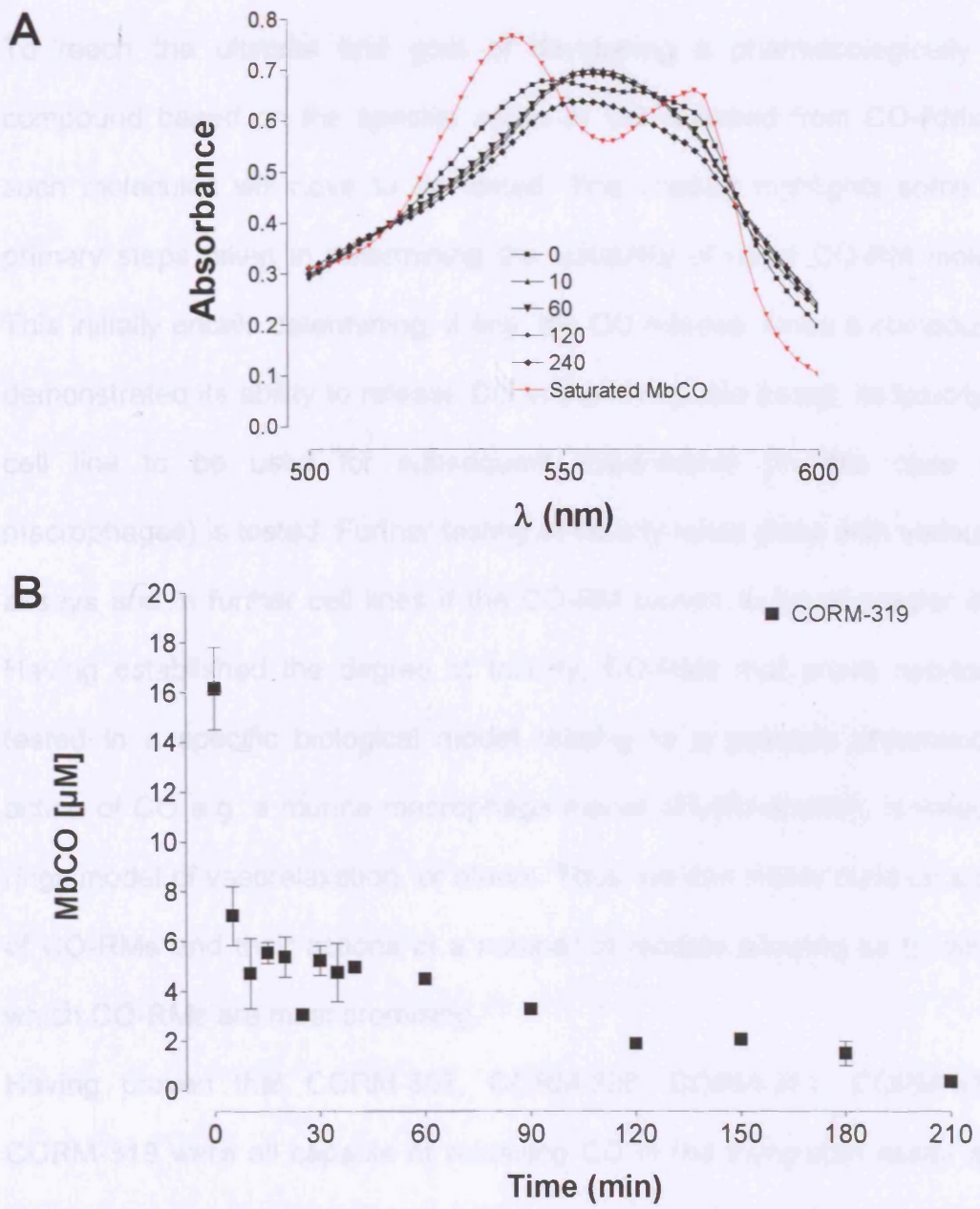


Figure 5.14:- Stability of CORM-319 in pure distilled water: effect on CO release.

(A) 60µM CORM-319 was added to a 66 µM myoglobin solution from a 12 mM CORM-319 stock. The conversion of myoglobin to MbCO was allowed for 10 min after which a reading was taken. This was repeated every 5 min for the first 40 min then at 60 min and every 30 min thereafter. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CO-RM to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentrations were derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

5.5 Discussion

To reach the ultimate end goal of developing a pharmacologically active compound based on the specific action of CO released from CO-RMs many such molecules will have to be tested. This chapter highlights some of the primary steps taken in determining the suitability of novel CO-RM molecules. This initially entails determining, if any, the CO release. Once a compound has demonstrated its ability to release CO in the myoglobin assay, its toxicity in the cell line to be used for subsequent experiments (in this case murine macrophages) is tested. Further testing of toxicity takes place with various other assays and in further cell lines if the CO-RM proves to be of greater interest. Having established the degree of toxicity, CO-RMs that prove non-toxic are tested in a specific biological model relating to a possible pharmacological action of CO e.g. a murine macrophage model of inflammation, isolated aortic rings model of vasorelaxation, or others. Thus, we can slowly build up a potfolio of CO-RMs and their actions in a number of models allowing us to determine which CO-RMs are most promising.

Having proven that CORM-307, CORM-308, CORM-311, CORM-312 and CORM-319 were all capable of releasing CO in the myoglobin assay all were tested to determine their toxicity. This chapter is representative for the many CO-RMs that have been tested and only a small selection of CO-RMs are actually shown (see appendix 11.1 for the full list). We found that CORM-307, CORM-308 and CORM-312 could be immediately excluded from further investigation since they all proved to be highly toxic in the macrophage cell line used. This toxicity can not be attributed to the methanol used to solubilise the CO-RMs since CORM-311 is also solubilised in methanol and is not toxic. Due

to the solubility of CORM-319 in water its effect on cell viability was determined up to 1000 μM. Both CORM-319 and iCORM-319 had very silimar effects on cell viability with no observable loss at 10 to 100 μM.

Interestingly, CORM-311 and CORM-319 had no effect on HO activity (10-100 μM). This is useful to know since an increase in HO activity could be responsible for actions attributed to the CO released by both CO-RMs.

LPS induced nitrite generation in murine macrophages was attenuated by CORM-311 and CORM-319. CORM-311 caused a potent reduction in nitrite levels; however, without a negative control (iCO-RM version) we can not be certain as to whether the compound itself or the CO released is responsible for the observed effect. CORM-319 although not quite as effective as CORM-311 did also significantly reduced nitrite levels at both 50 and 100 μM. The ineffectiveness of iCORM-319 and the fact there is no increase in HO activity with CORM-319 strongly implicates CO as the mediator of this function.

Owing to its poor solubility in ethanol, the fact its not water soluble and that there is no iCO-RM version available CORM-311 was not tested any further. This is not to say it will not be used ever again but that it is a good compound for the chemists to develop further by ironing out some of the problems associated with its use, which is the purpose of this methodology of testing.

Being the most promising compound so far, CORM-319 was tested for toxicity in a number of cell viability assays. In both LDH and Typan blue assays CORM-319 displayed little observable reductions in viability which is in agreement with data obtained with the Alamar blue assay. Interestingly, iCORM-319 is significantly toxic in both the LDH and Trypan blue assay at concentrations of 500 μM and above. This intriguingly does not correlate with Alamar blue data

showing at 750 μM there is only a 20 % loss in viability while the Typan blue assay indicates ~95 % of cells are dead and the LDH assay inferred cytotoxicity is ~40 %. In spite of the data there is a general trend towards iCORM-319 concentrations of 500 μM and above being detrimental to the cell membrane integrity and structure. The lack of effect with CORM-319 suggests the inactive version is chemically quite different and as such it would be beneficial to try and identify its exact structure to better understand its chemistry and, therefore, the biological effects. Despite this, toxicity in the 10 – 100 μM range of both CORM-319 and iCORM-319 was insignificant.

Having already demonstrated CO release by CORM-319 in Chapter 4 a number of other factors affecting CO release were investigated. Intitally, the effect of increasing concentrations of CORM-319 was determined. It was found that CO release was concentration dependent and that the concentration of CORM-319 added was very similar to the MbCO concentration attained suggesting 1 μmole of CO per 1 μmole of CORM-319 is generated. Also of note was the fact that 10, 20 and 40 μM CORM-319 result in a gradual conversion of deoxyMb into MbCO while 60 μM elicits quite a striking increase in the rate saturating myoglobin in only 30 min. However, when assessing the stability of CORM-319 in water it was surprising to find that within 5 min ~50 % of its CO releasing ability is lost and in less than 3.5 h the CO loss is approximately 99 %. The instability of CORM-319 in water has important implications for its use in research and any future therapeutic applications. Preparation of CORM-319 requires it to be administered immediately and thus makes its use complicated. As mentioned previously, CORM-319 in powder form is stable in air for a couple of months.

5.6 Conclusion

The systematic testing and evaluation of novel CO-RMs is vital to establishing a core group of compounds capable of eliciting benefical biological effects through the action of CO. The basic protocol set out in the last 2 chapters allows the quick identification of promising candidates and highlights CO-RMs that although not perfect can be redesigned to produce a more biocompatible version (i.e. CORM-311). In this instance CORM-319 is a promising contender but issues with its stability require resolving before it can be investigated as a potential drug to deliver CO for therapeutic purposes.

6 Chapter 6: CORM-A1: a newly discovered CO-releasing compound

6.1 Introduction

In the search for additional compounds capable of liberating CO but with a chemical structure different to the transition metal carbonyls used thus far, our group identified sodium boranocarbonate ($Na_2[H_3BCO_2]$) (CORM-A1), the structure of which can be seen in Figure 6.1.

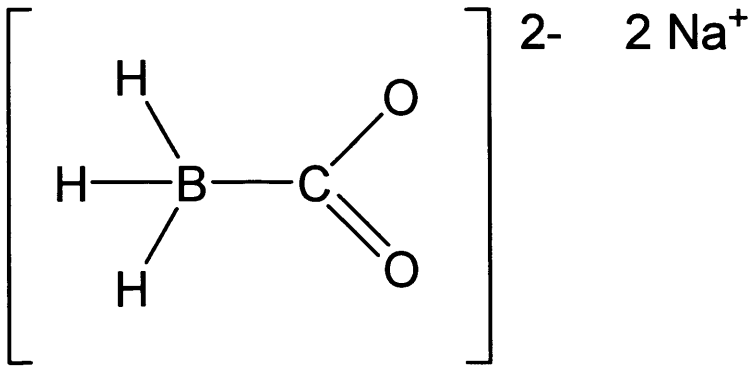


Figure 6.1:- Structure of CORM-A1. A non-metal containing CO-RM.

The idea of testing a boranocarbonate as a potential CO-RM originated from an interesting publication reporting the necessity of a CO source to synthesize a transition metal carbonyl ($[^{99m}Tc(OH_2)_3\text{-}(CO)_3]^+$) specifically designed for radio-imaging technology. This carbonyl can be prepared in a single-step procedure from aqueous $[^{99m}TcO_4]^-$ in the presence of CO gas and sodium borohydride as a reducing agent. However, the published preparation of $[^{99m}Tc(OH_2)_3\text{-}(CO)_3]^+$ relying on gaseous CO was unsuitable for use in commercial

radiopharmaceutical kits (Zhou et al. 1987; Kothari et al. 2000). The solution to this problem has been presented in a recent study reporting the first commercially feasible preparation of $[^{99m}Tc(OH_2)_3\text{-}(CO)_3]^+$ in physiological media using a boron-based carbonylating agent, potassium boranocarbonate, which acts as a CO source and a reducing agent simultaneously (Alberto et al. 2001). CORM-A1 does not contain a transition metal carbonyl but a carboxyl group (COO^-) covalently bound to the boron atom. At room temperature, it is known that aqueous solutions of CORM-A1 are alkaline and very stable but in the presence of hydrogen ions the compound starts to decompose and liberate CO. As with the other CO-RMs the release of CO and the toxicity had to be established prior to investigating its biological activities. In this chapter we again concentrate on the effect of CO on LPS induced nitrite generation in a murine macrophage model of inflammation.

6.2 Objective

Determine the CO releasing profile and bioactive properties of CORM-A1, a newly identified CO generator that does not contain a transition metal.

6.3 Experimental Protocol

The following section details the protocol of specific experiments of which more precise methods can be found in the Material and Methods chapter. Stock solutions of CORM-A1 (10 mM) were freshly prepared before each experiment by dissolving the compound in pure distilled water. CORM-A1 was layered with N_2 gas prior to returning it to storage in the freezer at -20°C.

6.3.1 Chemicals and reagents

Sodium boranocarbonate (CORM-A1) and Tricarbonylchloro-(glycinato)ruthenium (II) ([$Ru(CO)_3Cl$(glycinate)] or CORM-3) were synthesised as previously described (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b; Foresti, Hammad, Clark, Johnson, Mann, Friebe, Green, & Motterlini 2004; Alberto et al. 2001). CORM-A1 and CORM-3 were freshly prepared as 10 mM stock solutions in pure distilled water. CORM-A1 was inactivated (iCORM-A1) by adding a single drop of 1 M HCL to the CORM-A1 stock and bubbling the solution with nitrogen (N_2) gas for 5 min to remove any CO trapped in solution. The addition of acid caused a vigorous release of CO much like the initial release of gas from a carbonated drink. Inactive CORM-3 (iCORM-3) was also used in some experiments and was prepared by adding the compound to cell culture medium and leaving it for 18 h at 37 °C in a 5% CO_2 humidified atmosphere to liberate CO. The iCORM-3 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. Lipopolysaccharide (LPS - *E.Coli* serotype 026:B6) was obtained from Sigma. Polyclonal antibodies against HO-1 were from Bioquote Ltd (York, UK). Antibodies against β-actin were purchased from Abcam (Cambridge, UK). All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

6.3.2 Myoglobin Assay

To prepare a stock of CORM-A1 for the myoglobin assay, 1 mg of CORM-A1 was added to 100 µl of dH_2O to give a 96 mM stock. From this 4, 8 and 12 mM working stocks were prepared. The myoglobin was warmed (37 °C), sodium

dithionite (0.1 %) added and a 1 ml sample of the myoglobin measured for the deoxy-Mb curve. This 1 ml sample of deoxy-Mb was then bubbled with CO gas for 10 seconds to saturate the myoglobin and give a MbCO curve. Six cuvettes were then loaded into the spectrophotometer carousel with 1 ml of deoxy-Mb in each, 5 µl of 4, 8, and 12 mM CORM-A1 working stocks were then added in duplicate to each cuvette. The resultant final concentrations of CORM-A1 being 20 (from 4 mM), 40 (from 8 mM) and 60 µM (from 12 mM). The myoglobin was then mixed gently using a pipette and layered with 500 µl of mineral oil. The samples were then read at set intervals for a set period of time. The release of CO from CORM-A1 was also determined at room temperature (25°C). When investigating the effect of pH on the release of CO by CORM-A1 myoglobin was prepared in PBS in which the pH had been altered to pH = 5.5, pH = 6.5, pH = 7.0, pH = 7.4 and pH = 8.0. Standard pH = 7.4 PBS was used and either 1 M HCL or 1 M KCl used to acidify or alkalise the PBS, repectively. CORM-A1 stability was determined by preparing the stock solution of CORM-A1 in either citrate (pH = 6.6) or saline (pH = 6.6). From these stocks 40 µM CORM-A1 was added to myoglobin and left to release CO for 10 min at 37 °C after which the spectra was read. This was repeated at 30 min and 60 min using the original stocks.

6.3.3 Cell Culture

Murine RAW264.7 monocyte macrophages, Girardi (human heart) cells, wild type smooth muscle cells (European Collection of Cell Cultures - Salisbury, Wiltshire, UK) and bovine aortic endothelial cells (Coriell Cell Repositories - Camden, NJ, USA) were cultured as per Material and Methods. Cultures were

maintained at 37 °C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

6.3.4 Experimental Protocol

Macrophages were exposed for 24 h to LPS (1 μg/ml) in the presence or absence of CORM-A1 (10, 50, 100 and 500 μM) and nitrite levels were determined at the end of the incubation. In a similar set of experiments, cells were preincubated for 3 h with LPS (1 μg/ml) prior to the addition of CORM-A1 (50, 100 and 200 μM) in the presence or absence of LPS (1 μg/ml) and nitrite measured at 18 h. The production of nitrite was also assessed in cells exposed simultaneously to LPS (1 μg/ml), CORM-A1 (10, 50 and 100 μM) and iCORM-3 (100 μM) for 24 h. Further experiments were carried out in which RAW264.7 macrophages were pre-incubated with CORM-A1 (100 μM) prepared in PBS at various pH (6.0, 6.5, 7.0 and 7.4) for 30 min after which the PBS was replaced with medium containing 100 μM CORM-A1 in the presence or absence of LPS (1 μg/ml) and incubated for a further 24 h after which nitrite production was assessed. For comparison CORM-3 (100 μM) was pre-incubated for 30 min in PBS (pH = 7.4) after which the PBS was replaced with medium containing CORM-3 (100 μM) in the presence or absence of LPS (1 μg/ml) for a further 24 h. Experiments were repeated with the negative control iCORM-A1 to assess whether the effects observed were due to the CO liberated by the CO-RM or caused by other components of the molecule. The effect of CORM-A1 and its inactive counterpart on the haem oxygenase pathway was also investigated. Specifically, bovine aortic endothelial cells, murine macrophages, wild type smooth muscle cells and Girardi cells were treated for 6 or 24 h in the presence

of 10, 50, 100 and 500 μM CORM-A1 and haem oxygenase activity as well as HO-1 protein expression determined.

6.3.5 Assay for Nitrite Levels

Nitrite levels were determined using the Griess method. The measurement of this parameter is widely accepted as indicative of NO production. Briefly, the medium from treated cells cultured in 24 well plates was removed and placed into a 96 well plate (50 μl per well). The Griess reagent was added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0 μM to 300 μM in cell culture medium).

6.3.6 Western Blot Analysis

Samples of RAW264.7 cells were analysed by Western immunoblot. Briefly, an equal amount of protein (30 μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Bioquote Ltd., York, UK) (1:1000 dilution in Tris-buffered saline, pH = 7.4). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualised using an amplified alkaline phosphatase kit from Sigma (Extra-3A. To verify equal loading, samples were also probed with β-actin polyclonal antibodies (Abcam, Cambridge, U.K.).

6.3.7 Assay for Haem Oxygenase Activity

Haem oxygenase activity was determined in cells after various treatments. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH = 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 μM). The reaction was conducted at 37 °C in the dark for 1 h and terminated by the addition of 1 ml chloroform; the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40 mM^{-1}cm^{-1}$).

6.3.8 Cell Viability

Alamar Blue

Cell metabolism was determined using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK). The assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised (blue) form to a reduced (red) form. The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control.

Trypan Blue

The status of the cell membrane was investigated using Trypan blue. The medium of treated cells is removed and the cells then washed with PBS 10X. Then a 1:1 solution of PBS (10X) and Trypan blue dye was added to cells. The

dye permeates cell in which the membrane has been compromised. By counting the number of cell dyed compared to those which are not the number of viable cells can be determined.

Lactate Dehydrogenase

Damage of the phopholipid bi-layer of cells was investigated using an LDH assay kit and carried out according to the manufacturer's instructions (Roche, UK). The medium of treated cells was collected, spun to remove any cellular debris and then a reaction mixture containg an INT dye added. LDH activity reflects its release from the cell membrane and a loss of viability.

6.3.9 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$.

6.4 Results

6.4.1 CORM-A1 CO release is dependent on concentration, pH and temperature.

As with the CO-RMs tested thus far, the first priority was to establish the release of CO from CORM-A1. CO release from CORM-A1 in the myoglobin assay is slow and gradual compared to CO-RM-3 but shares a similar release to CORM-319 (Figure 6.2D). The CO release is concentration-dependent but each concentration of CO-RM fails to yield an equilivant to the amount put in, suggesting less than a 1:1 return on CORM-A1 to CO. The conversion of deoxyMb to MbCO over time at each concentration is shown in Figure 6.2A, Figure 6.2B and Figure 6.2C. Note the gradual change from a single peak spectra in Figure 6.2B to the double peak associated with MbCO. As predicted, iCORM-A1 was unable to convert the myoglobin to MbCO (Figure 6.2D).

From its chemistry we predicted that CORM-A1 would be unstable in the presence of protons (H^+) which should accelerate the release of CO. To investigate this we tested CORM-A1 in myoglobin prepared with PBS solutions of varying pH (Figure 6.3). Important to note is that the myoglobin spectra of both deoxyMb and MbCO was unchanged by the different pH. As pH was increased from pH = 5.5 to pH = 8.0 there is a striking change in the spectra. We see that CORM-A1 at low pH almost instantaneously releases and saturates the myoglobin, while increasing the pH results in a much more prolonged CO release and conversion of the spectra from the single peaked deoxyMb curve to the double peaked MbCO spectra (Figure 6.3A, Figure 6.3B, Figure 6.3C, Figure 6.3D and Figure 6.3F). This significant increase in the rate

of release is better displayed in Figure 6.4 which shows the MbCO concentrations.

Changes in pH result in the greatest change in rate from pH = 7.0 to pH = 7.4 where near instantaneous release suddenly becomes gradual. Further increasing pH seems to affect the CO release little possibly suggesting there is an all or nothing like switch between pH = 7.0 and pH = 7.4 where the proton concentration is enough to stimulate an immediate release.

Interestingly, we also show that temperature has some role in the rate of CO release (Figure 6.3E and Figure 6.4). Performing the myoglobin assay at room temperature results in a markedly decreased rate of CO release compared to 37 °C (Figure 6.3D and Figure 6.3E). Overall the end point is similar but the time taken to reach 'saturation' is more than doubled. This could possibly be related in some part to pH since pH is dependent on temperature.

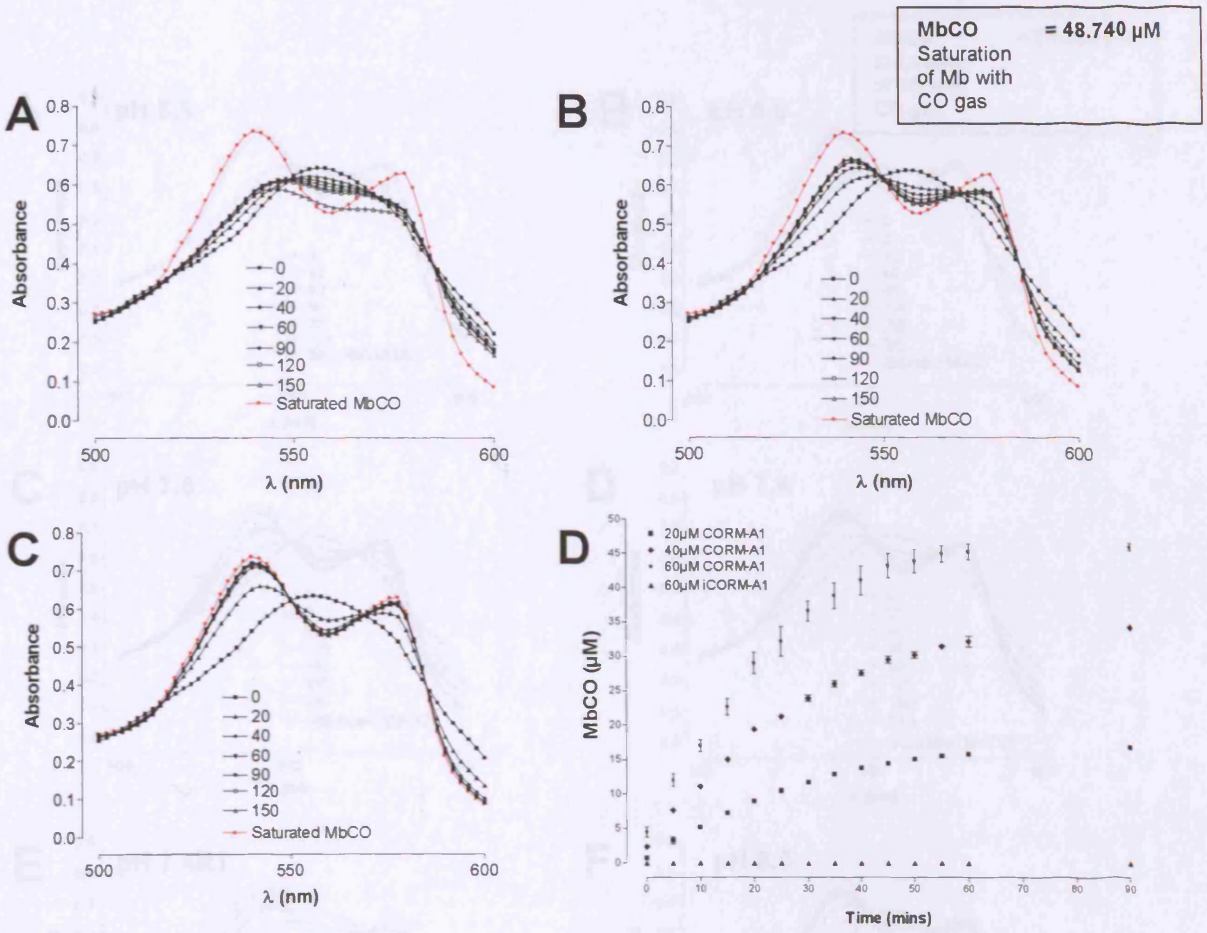


Figure 6.2:- Effect of increasing concentrations of CORM-A1 on myoglobin CO saturation.

(A) 20 µM CORM-A1 (B) 40 µM CORM-A1 and (C) 60 µM CORM-A1 was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-A1 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (D) The MbCO concentrations were derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

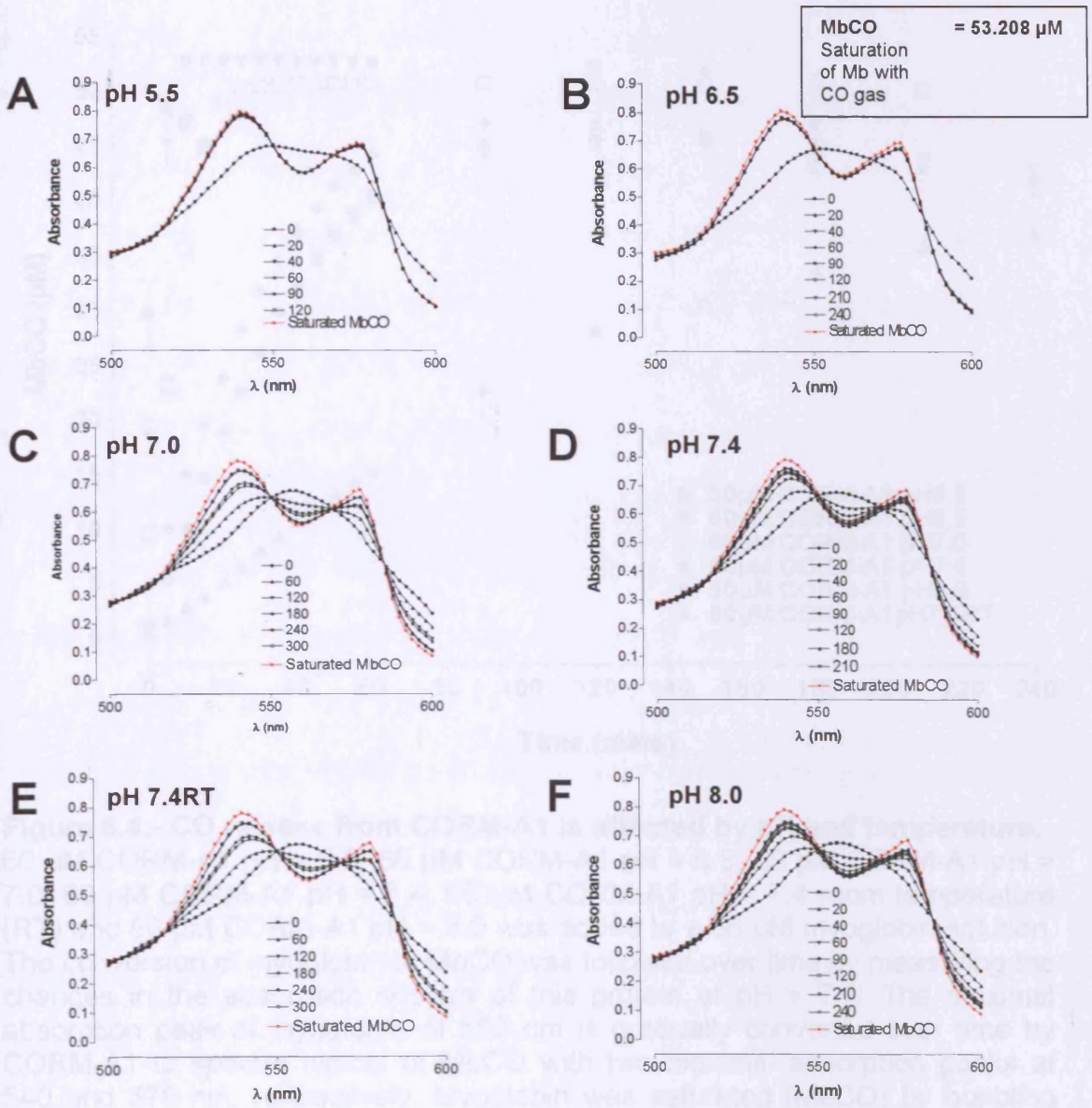


Figure 6.3:- Conversion of deoxy-myoglobin to carboxy-myoglobin by CORM-A1 in myoglobin of varying pH.

(A) 60 µM CORM-A1 pH = 5.5, (B) 60 µM CORM-A1 pH = 6.5, (C) 60 µM CORM-A1 pH = 7.0, (D) 60 µM CORM-A1 pH = 7.4, (E) 60 µM CORM-A1 pH = 7.4 room temperature (RT) and (F) 60 µM CORM-A1 pH = 8.0 was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-A1 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min.

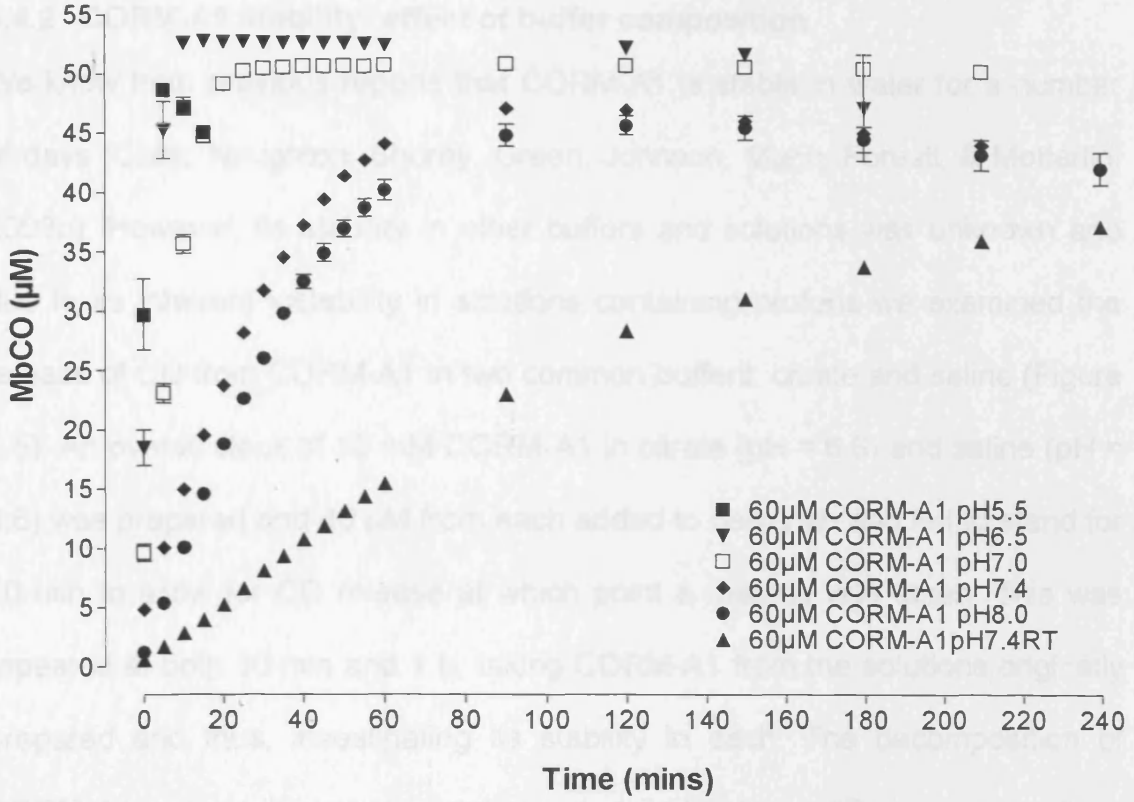


Figure 6.4:- CO release from CORM-A1 is affected by pH and temperature. 60 μM CORM-A1 pH = 5.5, 60 μM CORM-A1 pH = 6.5, 60 μM CORM-A1 pH = 7.0, 60 μM CORM-A1 pH = 7.4, 60 μM CORM-A1 pH = 7.4 room temperature (RT) and 60 μM CORM-A1 pH = 8.0 was added to a 66 μM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-A1 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. The MbCO concentrations were derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

6.4.2 CORM-A1 stability: effect of buffer composition.

We know from previous reports that CORM-A1 is stable in water for a number of days (Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b). However, its stability in other buffers and solutions was unknown and due to its inherent instability in solutions containing protons we examined the release of CO from CORM-A1 in two common buffers: citrate and saline (Figure 6.5). An overall stock of 10 mM CORM-A1 in citrate (pH = 6.6) and saline (pH = 6.6) was prepared and 40 μM from each added to deoxyMb and left to stand for 10 min to allow for CO release at which point a reading was taken. This was repeated at both 30 min and 1 h, taking CORM-A1 from the solutions originally prepared and thus, investigating its stability in each. The decomposition of CORM-A1 was similar in both solutions, despite the large difference in readings at T_0 (Figure 6.5A, Figure 6.5B and Figure 6.5C). This experiment helps to highlight that when using CORM-A1 prepared in biological buffers and commonly used vehicles, CORM-A1 administration should be immediate otherwise the CO release will be greatly diminished.

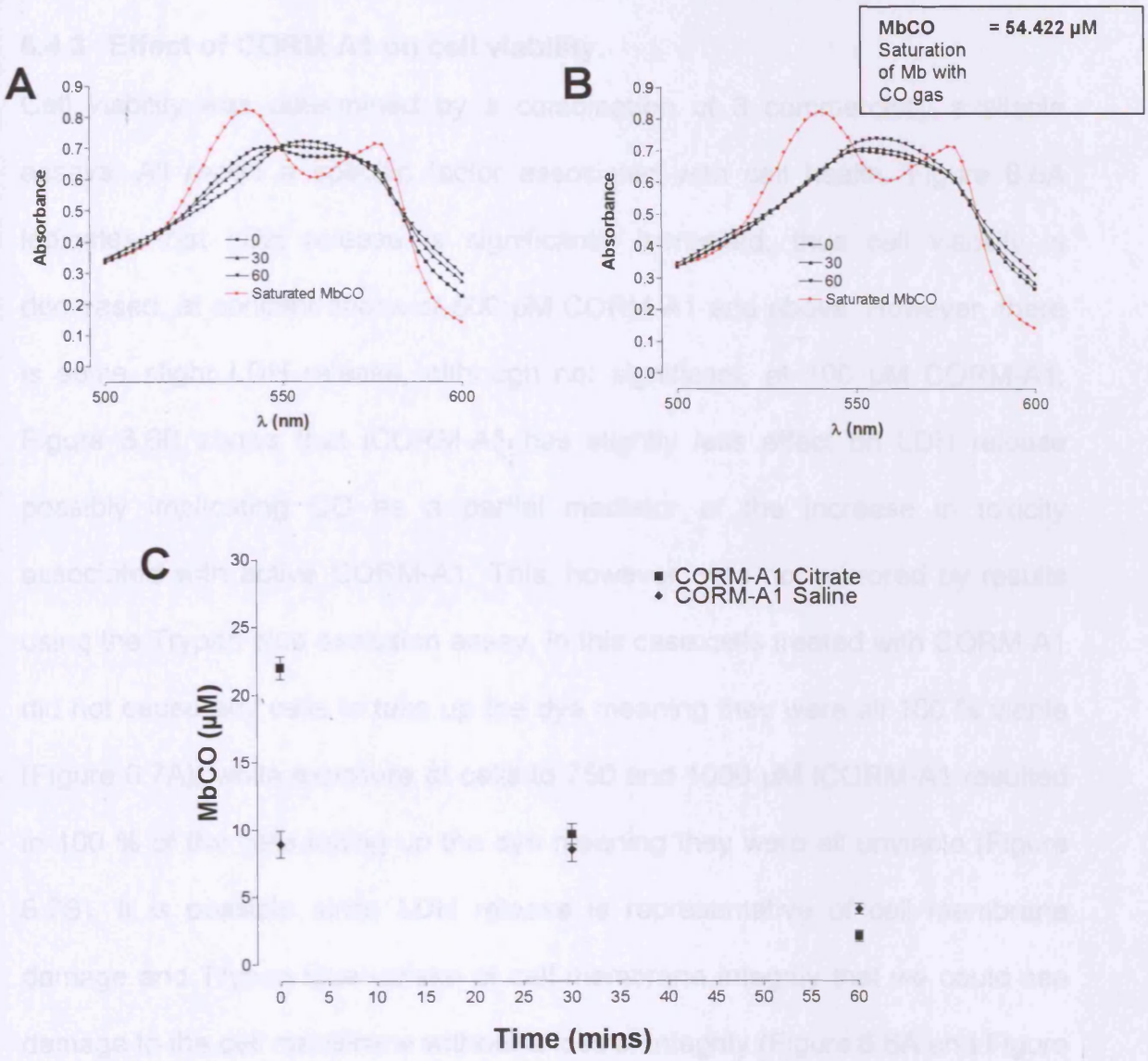


Figure 6.5:- Stability of CORM-A1 in myoglobin prepared with biological buffers.

(A) Stability of 10 mM CORM-A1 left to stand in a citrate solution pH = 6.6 for 60 min. (B) Stability of 10 mM CORM-A1 left to stand in a saline solution pH = 6.6 for 60 min. At 0, 30 and 60 min 40 µM CORM-A1 from either solution was added to a 66 µM myoglobin solution. The conversion of myoglobin to MbCO was then followed for 10 min by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-A1 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (C) The MbCO concentrations were derived from the increase in absorption between deoxyMb (not shown) and sample readings at 540 nm. This is the amount of CO released in solution.

6.4.3 Effect of CORM-A1 on cell viability.

Cell viability was determined by a combination of 3 commercially available assays. All report a specific factor associated with cell health. Figure 6.6A indicates that LDH release is significantly increased, thus cell viability is decreased, at concentrations of 500 μM CORM-A1 and above. However, there is some slight LDH release, although not significant, at 100 μM CORM-A1. Figure 6.6B shows that iCORM-A1 has slightly less effect on LDH release possibly implicating CO as a partial mediator of the increase in toxicity associated with active CORM-A1. This, however, was not mirrored by results using the Trypan blue exclusion assay. In this case cells treated with CORM-A1 did not cause any cells to take up the dye meaning they were all 100 % viable (Figure 6.7A), while exposure of cells to 750 and 1000 μM iCORM-A1 resulted in 100 % of the cells taking up the dye meaning they were all unviable (Figure 6.7B). It is possible since LDH release is representative of cell membrane damage and Trypan blue uptake of cell membrane integrity that we could see damage to the cell membrane without a loss of integrity (Figure 6.6A and Figure 6.7A). While, the damage caused by iCORM-A1 resulted in a dramatic loss of cell membrane integrity allowing the Trypan blue to enter the cell and LDH to be released in large volumes (Figure 6.6B and Figure 6.7B). The Alamar blue assay gives us an alternative to the cell membrane associated assays and relies on cell metabolism as an indicator of cell health. CORM-A1 in this instance seems to have less of an effect on metabolism than cell membrane components with only 1000 μM CORM-A1 causing any significant reduction in viability (Figure 6.8A). Interestingly, iCORM-A1 has no effect on metabolism at all (Figure 6.8B) which is in disagreement with both LDH and Trypan blue

assays which suggest a complete loss of viability (Figure 6.6B and Figure 6.7B). This justifies the need for multiple assays for cell viability as each has its own limitations. Indeed, Alamar blue relies upon the reduction of a dye to infer cellular metabolism but CORM-A1 is a known reducing agent which could potentially interfere with the assay (Voytik-Harbin, Brightman, Waisner, Lamar, & Badylak 1998; Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003b).

The most important aspect to note is that at the concentrations used most frequently (10–100 μM) there is no observable loss of viability in any of the three assays.

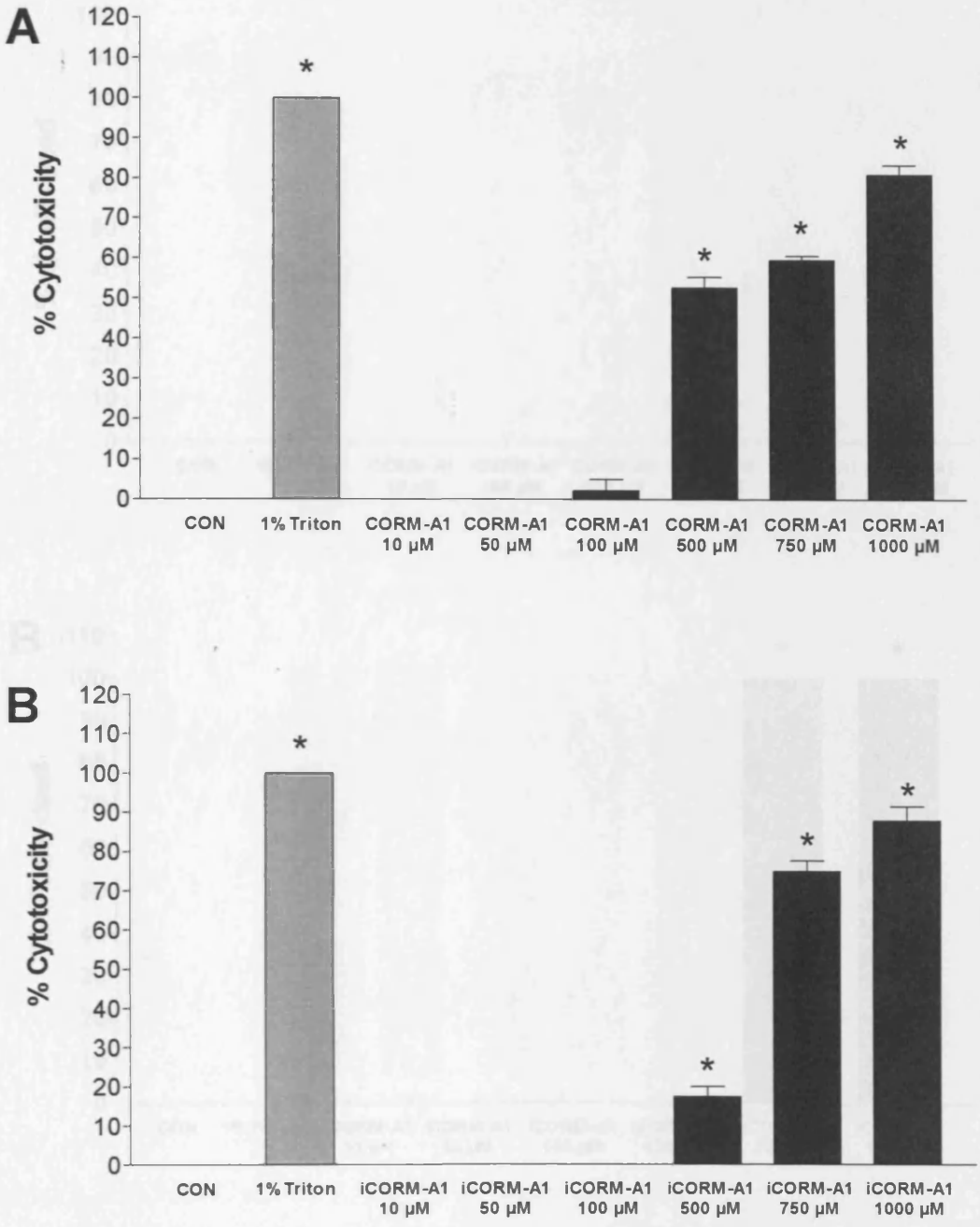


Figure 6.6:- The effect of CORM-A1 on LDH release in RAW264.7 macrophages.
The release of LDH was assessed 24 h after exposure of macrophages to (A) CORM-A1 (10-1000 µM) or (B) iCORM-A1 (10-1000 µM) using an LDH kit purchased from Roche Diagnostics. Viability was expressed as a percentage of maximal damage (triton) and minimal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates P< 0.005 vs. control.

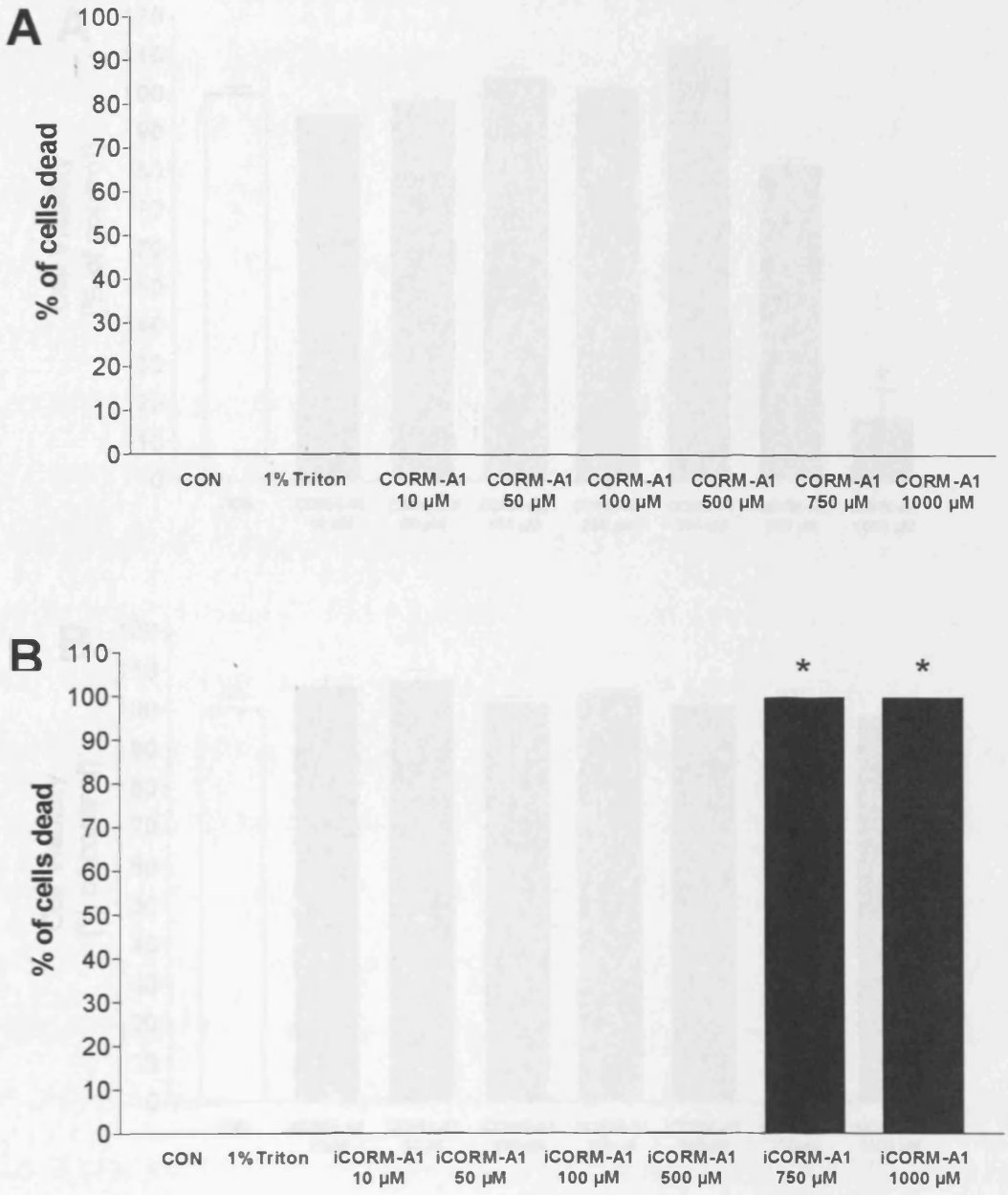


Figure 6.7:- The effect of CORM-3 on cell membrane permeability to Trypan blue dye.

Cell membrane permeability was assessed 24 h exposure of macrophages to (A) CORM-A1 (10-1000 µM) or (B) iCORM-A1 (10-1000 µM) using the Trypan blue exclusion assay. Viability was expressed as a percentage of the cells stained with the dye compared to the total number of cells. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

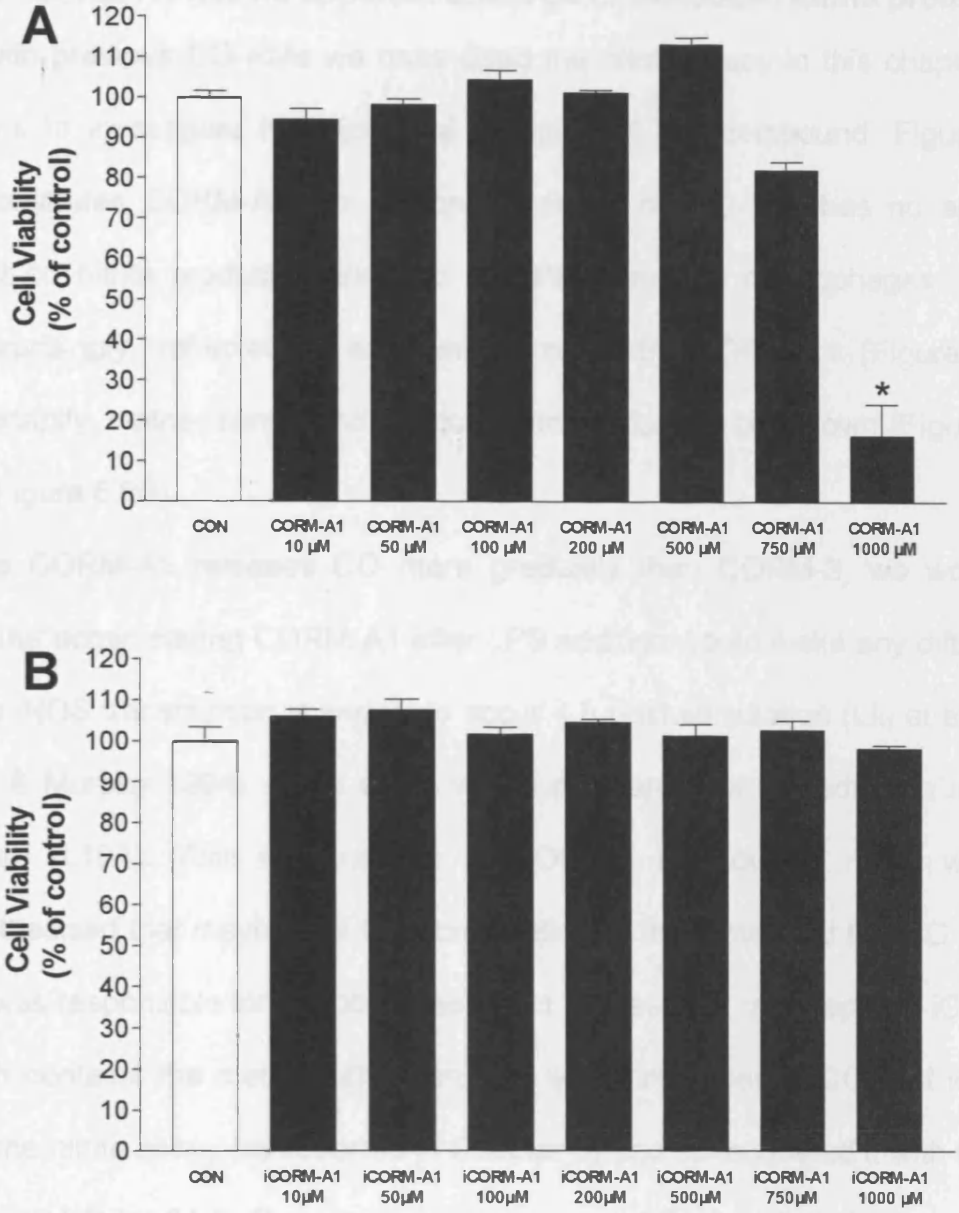


Figure 6.8:- The effect of CORM-3 on cellular metabolism.
Cell metabolism was assessed 24 h exposure of macrophages to (A) CORM-A1 (10-1000 µM) or (B) iCORM-A1 (10-1000 µM) using the Alamar blue viability assay. Viability was expressed as a percentage of control. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P<0.05$ vs. control.

6.4.4 CORM-A1 has no apparent effect on LPS-induced nitrite production.

As with previous CO-RMs we have used the nitrite assay in this chapter as a means to investigate the biological activities of this compound. Figure 6.9A demonstrates CORM-A1, up to concentrations of 500 μM, has no apparent effect on nitrite production induced by LPS in murine macrophages. This is, unsurprisingly, reflected in experiments run with iCORM-A1 (Figure 6.9B). Importantly, neither compound affects nitrite production on its own (Figure 6.9A and Figure 6.9B).

Since CORM-A1 releases CO more gradually than CORM-3, we wondered whether administering CORM-A1 after LPS addition would make any difference, since iNOS transcription is known to occur 4 h post stimulation (Liu et al. 1993; Park & Murphy 1994). Once again we found there was no reduction in nitrite (Figure 6.10A). With the success of CORM-3 in reducing nitrite we then hypothesised that maybe it is the combination of the metal and the CO release that was responsible for the observed effect. To test this, we prepared iCORM-3 which contains the metal, ruthenium, but would not release CO and interfere with the nitrite assay (as reported in Chapter 5) and co-incubated it with CORM-A1 and LPS for 24 h. Once again, there was no effect on the nitrite generated (Figure 6.10B).

Since CORM-3 releases CO instantaneously, we then investigated whether it could be the gradual release of CO from CORM-A1 that in some way prevented it from decreasing nitrite. Having already shown in Figure 6.4 that pH could increase the rate at which CO is released by CORM-A1, we pre-incubated cells for 30 min with CORM-A1 and LPS in PBS of varying pH (6.0 to 7.4) to stimulate increased CO release. After 30 min, PBS was replaced with medium

containing the corresponding CORM-A1 and LPS concentrations so as not to overexpose the cells to PBS which can be toxic over extended periods. Cell viability was assessed and the cells remained viable but there was no detectable decrease in the amount of nitrite generated (Figure 6.11). For comparison, CORM-3 (100 μM) alone was also exposed to the same treatment and still elicited a significant reduction in nitrite production (Figure 6.11).

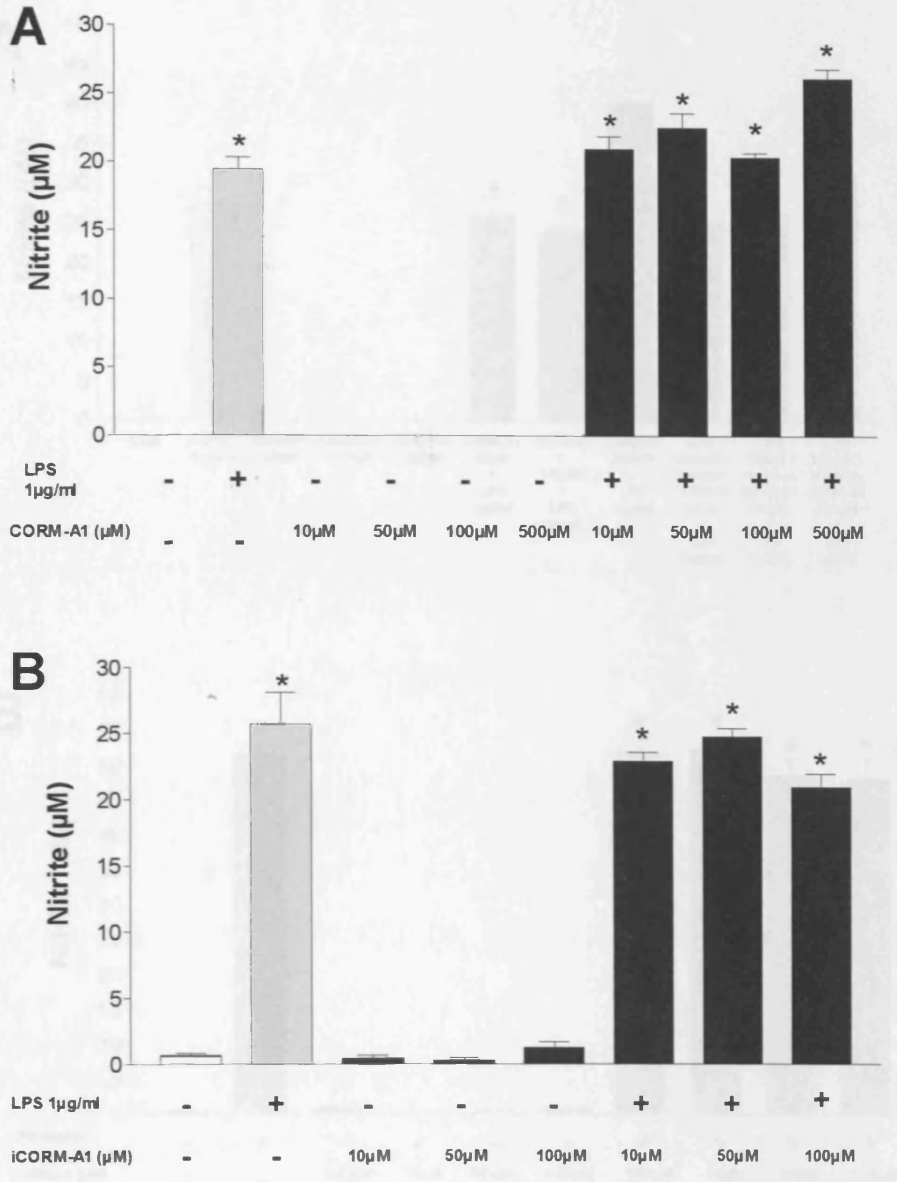


Figure 6.9:- Effect of CORM-A1 on LPS-stimulated nitrite production.
(A) RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-A1 (10-500 μM) and nitrite production was assessed at 24 h. (B) The inactive compound iCORM-A1 (0 - 500 μM) was also used to determine the contribution of CO released by CORM-A1 to the observed effect. Control cells were incubated with medium alone. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

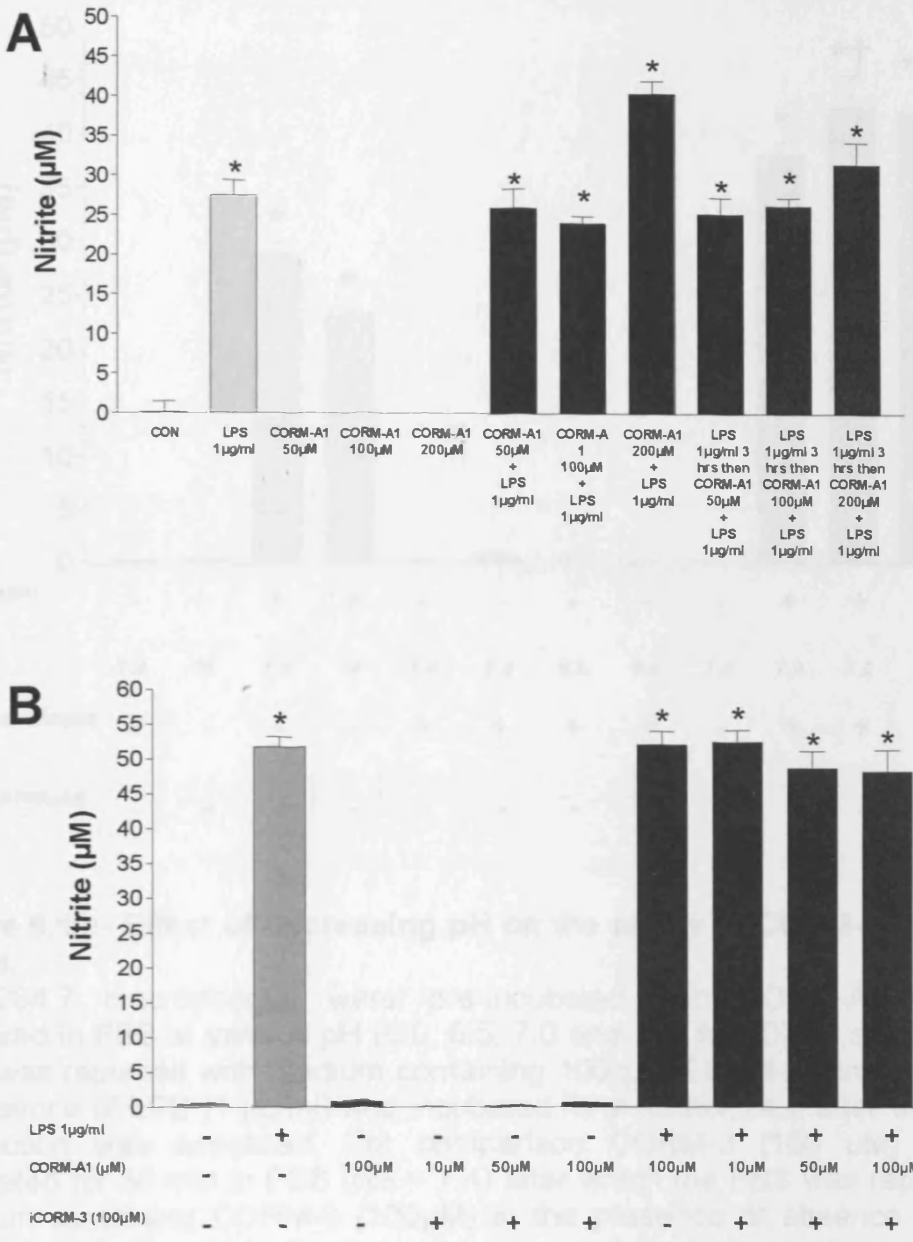


Figure 6.10:- Effect of LPS pre-incubation and co-incubation of iCORM-3 and CORM-A1 on LPS-stimulated nitrite production.

(A) RAW264.7 macrophages were either pre-incubated with 1 μg/ml LPS for 3 h prior to CORM-A1 addition or co-incubated with CORM-A1 (50-200 μM) and nitrite production was assessed at 18 h. (B) macrophage cells were incubated with 1 μg/ml LPS in the presence of both CORM-A1 (10-100 μM) and iCORM-3 (100 μM) after which nitrite was determined 24 h later. Control cells were incubated with medium alone. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

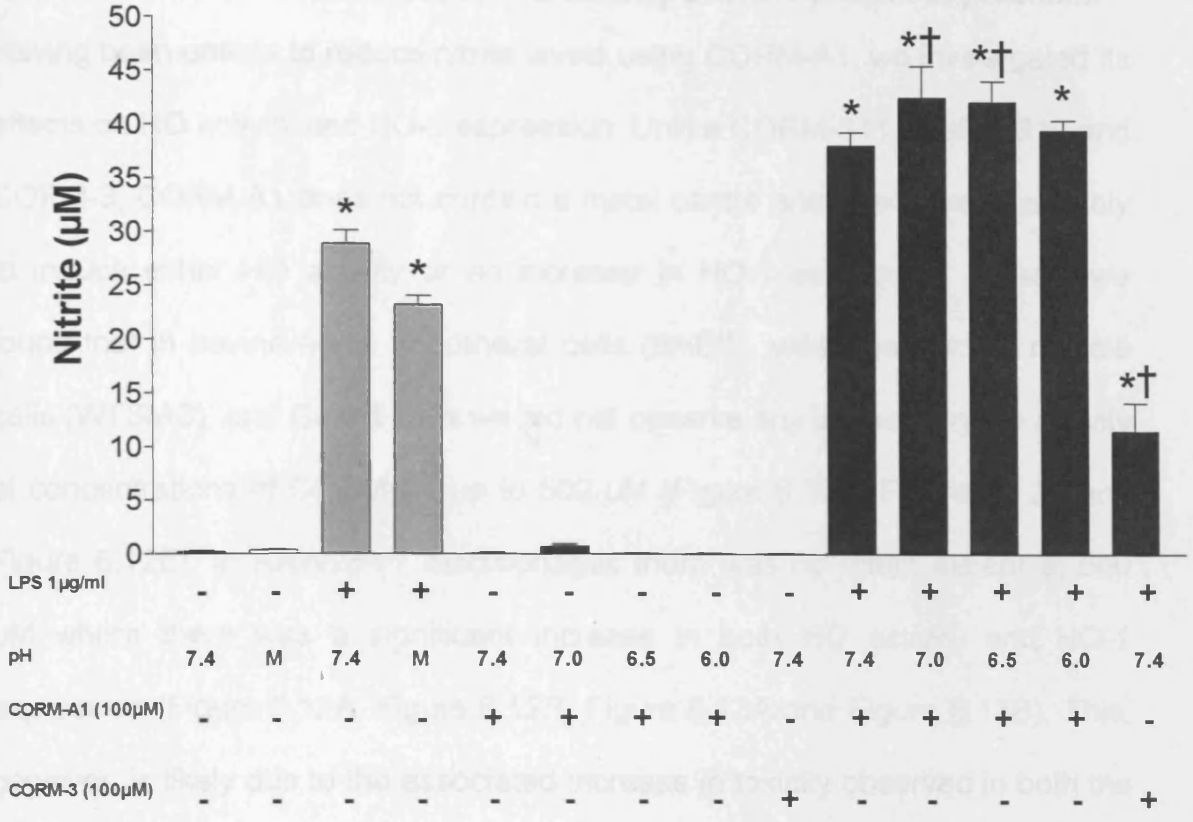


Figure 6.11:- Effect of decreasing pH on the ability of CORM-A1 to reduce nitrite.

RAW264.7 macrophages were pre-incubated with CORM-A1 (100 μM) prepared in PBS at various pH (6.0, 6.5, 7.0 and 7.4) for 30 min after which the PBS was replaced with medium containing 100 μM CORM-A1 in the presence or absence of LPS (1 μg/ml) and incubated for a further 24 h after which nitrite production was assessed. For comparison CORM-3 (100 μM) was pre-incubated for 30 min in PBS (pH = 7.4) after which the PBS was replaced with medium containing CORM-3 (100μM) in the presence or absence of LPS (1 μg/ml) for a further 24 h. Control cells were incubated with medium alone. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control; † indicates $P < 0.05$ vs. LPS (pH = 7.4).

6.4.5 CORM-A1 has no effect on HO activity or HO-1 protein expression.

Having been unable to reduce nitrite levels using CORM-A1, we investigated its effects on HO activity and HO-1 expression. Unlike CORM-311, CORM-319 and CORM-3, CORM-A1 does not contain a metal centre and, therefore, is unlikely to induce either HO activity or an increase in HO-1 expression. Indeed, we found that in bovine aortic endothelial cells (BAEC), wild type smooth muscle cells (WTSMC), and Girardi cells we did not observe any increase in HO activity at concentrations of CORM-A1 up to 500 μM (Figure 6.12C, Figure 6.12D and Figure 6.12E). In RAW264.7 macrophages there was no effect except at 500 μM where there was a significant increase in both HO activity and HO-1 expression (Figure 6.12A, Figure 6.12B, Figure 6.13A and Figure 6.13B). This, however, is likely due to the associated increase in toxicity observed in both the LDH assay (Figure 6.6A) and the Alamar blue assay (Figure 6.8A).

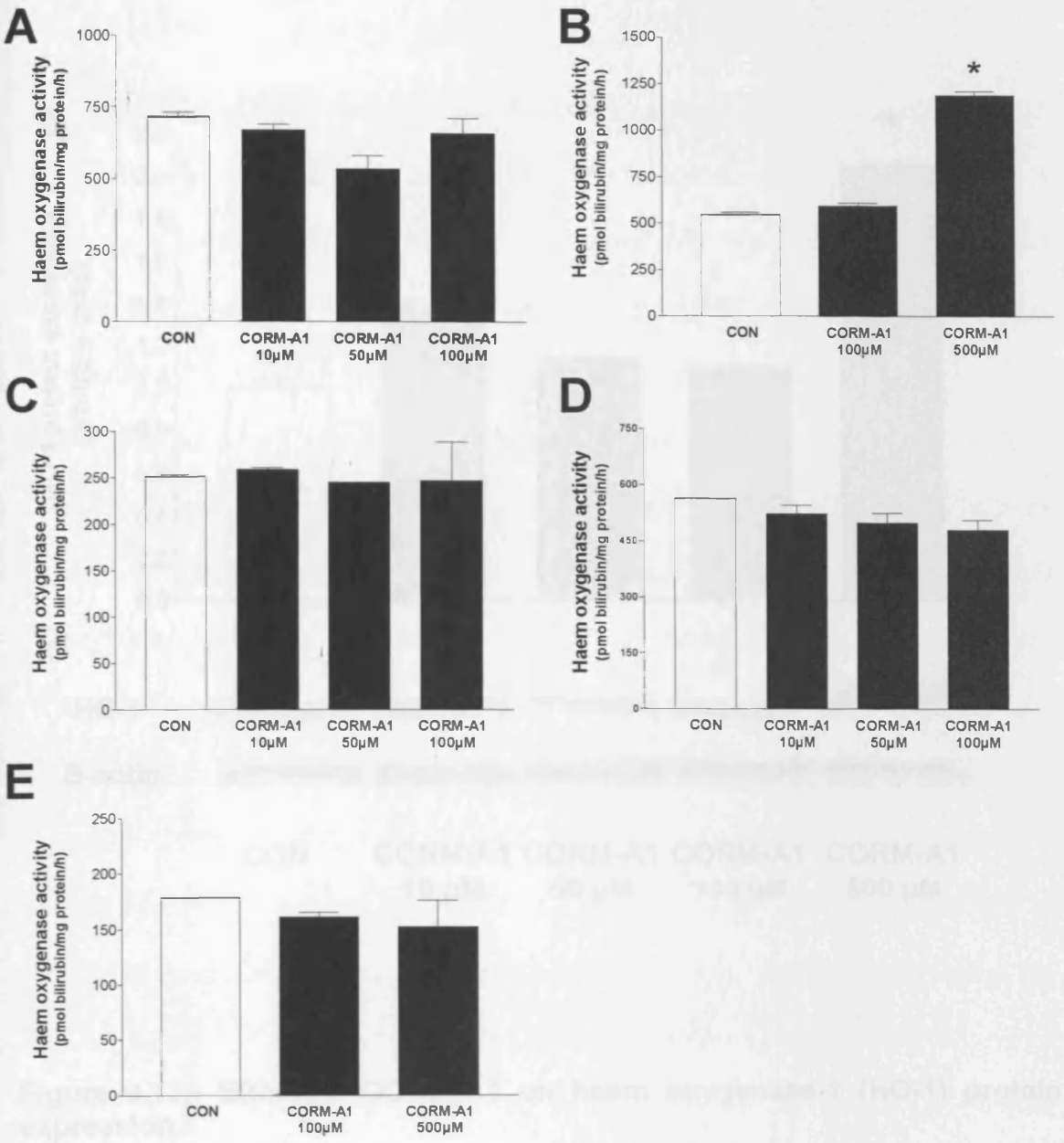


Figure 6.12:- Effect of CORM-A1 on haem oxygenase activity in various cell lines.

RAW264.7 macrophages were incubated with increasing concentrations of (A) CORM-A1 (10-100 μM) for 6 h and (B) CORM-A1 (100-500 μM) for 24h. (C) wild type smooth muscle cells and (D) Girardi cells were treated with CORM-A1 (10-100 μM) for 6 h. (E) while, bovine aortic endothelial cells were exposed to CORM-A1 (100-500 μM) for 24 h. Haem oxygenase activity was determined after exposure to the different treatments. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

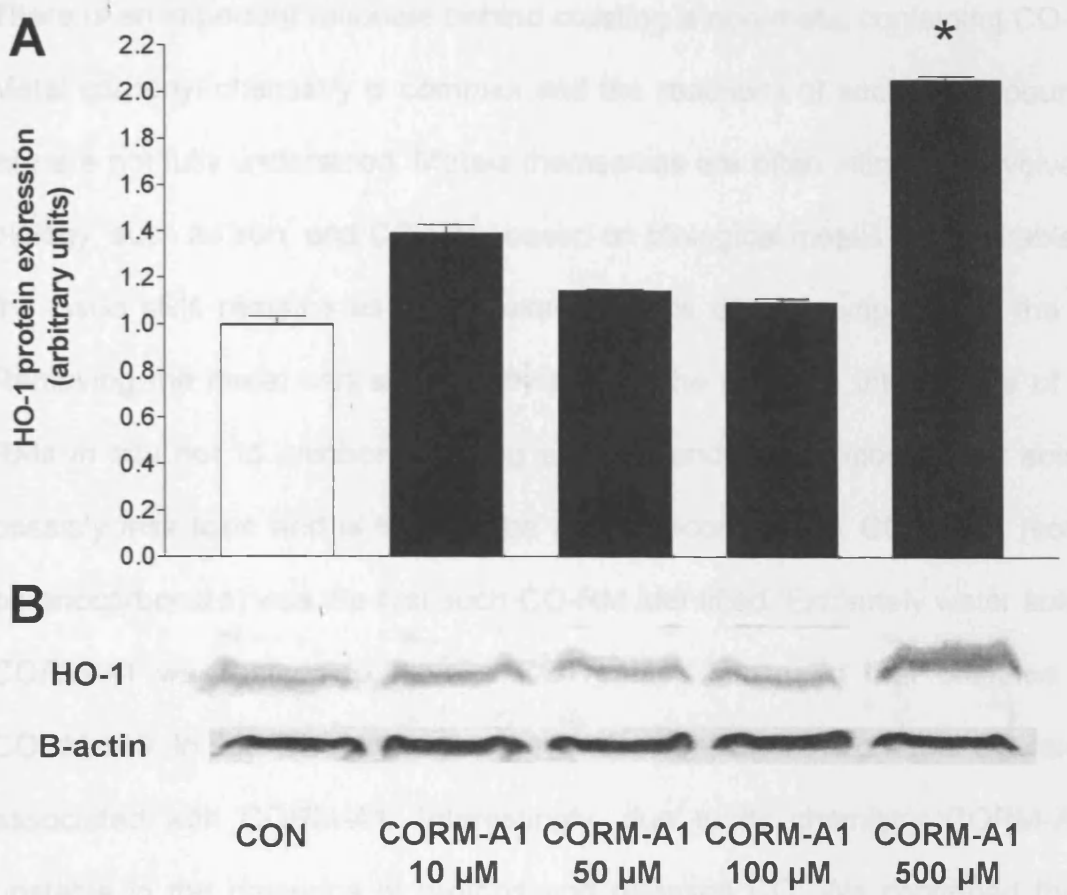


Figure 6.13:- Effect of CORM-A1 on haem oxygenase-1 (HO-1) protein expression.
RAW264.7 macrophages were incubated with increasing concentrations of CORM-A1 (10-500 µM) and HO-1 protein levels were determined by Western blot analysis after 24 h. (A) The graph shows the average expression of HO-1 following densitometric analysis of three different (B) blots from three independent experiments and one representative image is reported. β-actin was used as an internal control for equal loading. * indicates $P < 0.05$ vs. control.

6.5 Discussion

There is an important rationale behind creating a non-metal containing CO-RM. Metal carbonyl chemistry is complex and the reactions of such a compound *in situ* are not fully understood. Metals themselves are often intimately involved in biology, such as iron, and CO-RMs based on biological metals are desirable but the issue stills remains as to the exact actions of the compound in the cell. Removing the metal can significantly reduce the potential interactions of CO-RMs *in situ* not to mention creating a compound that is more water soluble, possibly less toxic and is likely to be more biocompatible. CORM-A1 (sodium boranocarbonate) was the first such CO-RM identified. Extremely water soluble, CORM-A1 was shown to have a CO release similar to that obtained with CORM-319. In this Thesis we have again demonstrated the gradual CO release associated with CORM-A1. Interestingly, due to its chemistry CORM-A1 is unstable in the presence of protons and releases CO. We confirmed this by running the myoglobin assay using myoglobin prepared with PBS at various pH. The increase in CO release at acidic pH is striking with near instantaneous release. It is interesting to note that the change in the rate of CO release between pH = 7.0 to pH = 5.5 is not particularly significant, although, between pH = 7.4 and pH = 7.0 there is a large increase in rate. This could suggest a switch like response where a small change in proton gradient results in a big increase in CO release but further increases in proton concentration have little affect on subsequent CO release. We also found that temperature plays an important role in CO release. CORM-A1 tested in the myoglobin assay at room temperature (25°C) has a considerably slower CO release than at 37 °C. This

could be as a result of the effect of temperature on pH, thus affecting the proton gradient (Kipp & Schuck 1995).

Having established the ability of CORM-A1 to release CO, next it was important to determine its toxicity in the murine macrophage cell line to be used for the nitrite study. We found that CORM-A1 at the concentrations most frequently used (10-100 μM) was not toxic in any of the assays. Interestingly, at higher concentrations (500-1000 μM), there was some disagreement between the cytotoxicity assays utilised. CORM-A1 elicited LDH release at 500 μM and above but did not affect Trypan blue and only affected Alamar blue at 1000 μM. Meanwhile, iCORM-A1 at 750 and 1000 μM was almost 100 % toxic in both the LDH assay and Trypan blue assay but did not affect Alamar blue at all. From the data obtained with these assays we can conclude that CORM-A1 at high concentrations seems to elicit some damage to the cell membrane enough to cause LDH release but not to actually split the cell membrane and also seems to affect the cellular metabolism probably as a direct result. Most importantly, no effect was seen in any assay at the most frequently used concentrations which have been known to cause a pharmacological effect in the vasculature (Motterlini et al. 2005).

Having determined the toxicity we then began to test the ability of CORM-A1 to reduce nitrite. Unlike CORM-3 we found CORM-A1 had effect on LPS-induced nitrite generation in murine macrophages. This we summised could be due to: i) the slower rate of CO release compared to CORM-3, ii) the absence of a metal centre, iii) the localisation of CO release. As such we devised a number of methods to explore these options with the exception of iii) which is a particularly complicated area of research and not feasible. We know that CORM-3 has an

instantaneous release of CO and that its actions appeared to be as a direct result of CO binding to iNOS and limiting its ability to generate nitrite. Having already established that the release of CO from CORM-A1 could be increased at acidic pH, we incubated cells in PBS at various pH containing CORM-A1. In theory, the accelerated CO release should result in CO levels equal to that of cells exposed to CORM-3. However, there was no reduction in nitrite as a result of this approach. Nor was it affected by delaying the addition of CORM-A1 until such time that iNOS would have been transcribed thus allowing CO release to occur concomitantly with the period of iNOS activity. Finally, cells were incubated with iCORM-3 and CORM-A1. The presence of the metal, we hypothesised, would in some way function to allow CO to reduce nitrite. This, however, also failed to achieve the desired result. This is most interesting since in our laboratory, *ex vivo* experiments showed that CORM-A1 promotes pharmacological activities. Indeed, CORM-A1 has been shown to have a significant vasodilatory effect in an isolated aortic ring model (Motterlini, Sawle, Hammad, Bains, Alberto, Foresti, & Green 2005). In this instance the effect would appear to involve endothelium independent relaxation that is partially mediated by sGC and does not require the activation of potassium channels, unlike CORM-3 which, is both endothelium-dependent and does require potassium channel activation. While it has also been shown (data unpublished) that CORM-A1 has a beneficial effect in a Langendorff model of ischemia reperfusion injury. Studies have also been carried out showing a vasodilatory effect of CORM-A1 on kidney function and modulation of oxygen saturation in an isolated mitochondria preparation. It was assumed a more gradual CO release would have more of an effect on inflammation due to the prolonged

nature of such an insult. It is possible that CORM-A1 acts in a different cellular location to CORM-3 and thus does not elicit a similar response. The exact mechanism of release and subsequent fate of the compound and the CO released are unkown and need to be investigated further to understand the difference between their actions.

Unlike CORM-3, CORM-A1 has also no effect on HO activity in a variety of cell types or HO-1 expression in murine macrophages. This is likely due to the absence of both a metal and the biological ligand haem precursor glycine. Thus, any biological activity attributed to CORM-A1 is not due to HO activity but due to the CO released or the compound itself.

6.6 Conclusion

There is no apparent effect of CORM-A1 on LPS induced nitrite generation in murine macrophages. The compound is not toxic between 10 and 100 μM and neither is the negative control (iCORM-A1). CO release is gradual and dependent on concentration, pH and temperatue. CORM-A1 has no effect on HO activity in various cell lines and no effect on HO-1 protein expression in murine macrophages. The compound seems well suited to be used in biological systems and its success in other systems should encourage further investigation in to the exact mechanism of its CO release in the cellular environment in an attempt to elucidate the differences between its actions and CORM-3.

7 Chapter 7: CORM-F: An Example of the Multiplicity of Metal Carbonyl Compounds

7.1 Introduction

In recent years our group has been testing and characterising metal carbonyl complexes, and more recently non metal compounds, as a means to release CO endogenously. Metal carbonyl complexes have long been used in chemistry to purify metals but their use in biological systems is novel, although quickly finding favour. These compounds have been used in areas as diverse as cancer therapy, drug receptor interactions and malarial research. Bio-organometallic chemistry has now become an emerging discipline that may offer innovative solutions to many biological problems.

These metal carbonyl complexes or carbon monoxide-releasing molecules (CO-RMs) are capable of releasing CO in the biological milieu and already have been shown to elicit numerous biological effects already attributed to CO such as anti-inflammatory, anti-hypertensive, anti-rejection and vasoregulatory activities. Interestingly, minor modifications of the chemical structure can allow for improved CO release, various rates of CO release, reduced toxicity and greater solubility.

By manipulating the ligands the rate at which CO is released can be altered, allowing for slow delivery of CO over a period of time or a faster release where needed. CO-RMs that release CO very rapidly ('fast releasers') in biological systems would be ideal for therapeutic applications where CO acts as a prompt signalling mediator (i.e. neurotransmission, acute hypertension, angina, ischemia-reperfusion). However, the discovery of chemicals that release CO

with a slow kinetic ('slow releasers') would implement the design of pharmaceuticals that could be more versatile in the treatment of certain chronic diseases (i.e. inflammatory states and chronic hypertension; rejection of transplanted organs) where the continuous and long-lasting effects of CO may be required. The attempt to diversify the multiplicity of CO-RMs that possess a variety of chemical characteristics (i.e. water vs. lipid-soluble; slow vs. fast releasers) will help elucidate the biological function of cellular targets that are responsive to CO and will facilitate in due course the design of versatile agents that could be used for the therapeutic delivery of CO in a safe and measurable fashion.

CORM-F compounds are a group of molecules designed by Dr Ian Fairlamb (University of York) based on 2-pyrones (See Figure 7.1). 2-pyrone (2H-pyran-2-one) is a ubiquitous structural motif found in nature (McGlacken & Fairlamb 2005). Both natural and synthetic 2-pyrone compounds have been found to exhibit a range of bioactivities such as the ability to inhibit human ovarian carcinoma (A2780) and human chronic myelogenous leukaemia (K562) cell lines at the sub-micromolar level (IC_{50}) (Moreno-Manas 1988; Marrison, Dickinson, & Fairlamb 2002).

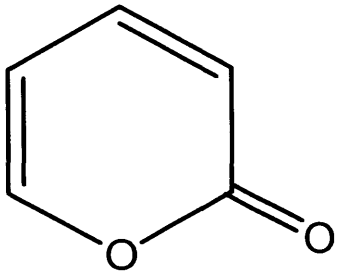


Figure 7.1:- The chemical structure of 2-pyrone

The action of 2-pyrone appears to be as a pro-drug where the opening of the carbonyl ring leads to the bioactive form (Fairlamb et al. 2004). 6-chloro-2-pyrones have been shown to inhibit yeast cholesterol esterase from Candida Cylindracea in a similar manner. Fairlamb and co-workers have since established a plethora of functionalised 2-pyrones as broad spectrum inhibitors of bacteria, fungi and yeasts – with inhibition being related to interference of sterol related biosynthetic pathways (Fairlamb, Marrison, Dickinson, Lu, & Schmidt 2004). It is predicted that the unique structural properties of the core molecule could exhibit interesting bioactivity in appropriately selected biological systems (Figure 7.2).

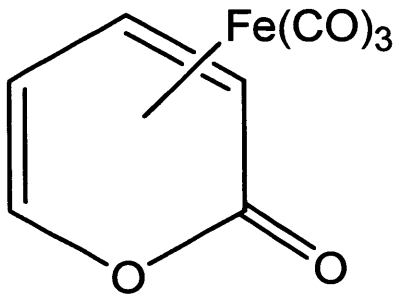


Figure 7.2:- Basic structure of CORM-F derivatives.

CORM-F compounds are a particularly good example of the malleable nature of CO-RMs which show just how minor adjustments can result in significant differences between compounds. Overall they are a good demonstration of the power of CO-RMs to be adapted to suit a desired action.

7.2 Objective

Demonstrate how structure can affect CO release, toxicity and nitrite using CORM-F compounds.

7.3 Materials and Methods

7.3.1 Chemicals and reagents

The CO-RMs tested in this chapter were prepared by Dr Ian Fairlamb (University of York). CO-RMs were prepared as 10 mM stock solutions in DMSO and used the same day of the experiment. The structures of all the CO-RMs are represented in Figure 7.3. All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

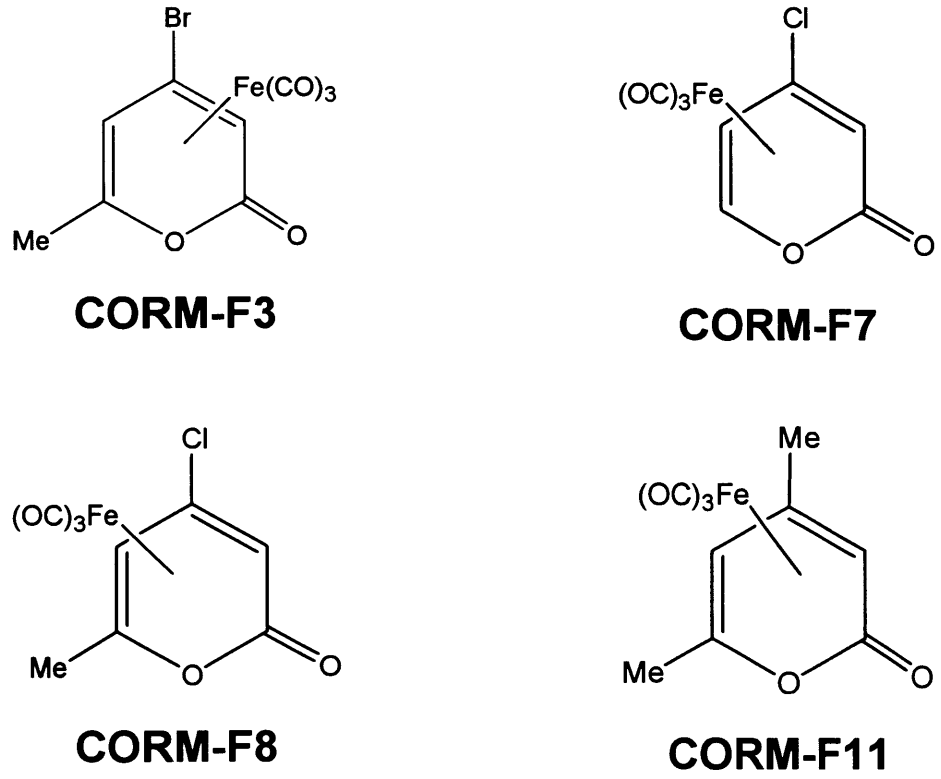


Figure 7.3:- The structure of CORM-F compounds in this chapter.
Note the overall structure of the compounds is identical. Variations comprise the addition/subtraction of a methyl group or altering the halogen atom.

7.3.2 Detection of CO Release

The release of CO from CO-RMs (40μM) was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO).

7.3.3 Cell Culture

Murine RAW264.7 monocyte macrophages (European Collection of Cell Cultures - Salisbury, Wiltshire, UK) were cultured as per Material and Methods. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

7.3.4 Experimental Protocol

Macrophages were treated for 24 h with 10, 50, 100 and 200 μM CO-RM (F3, F7, F8, and F11) in 24 well plates. After which the medium was collected for the lactate dehydrogenase assay, while the cells were used for the Alamar blue assay. The effect of CORM-F3 on nitrite levels was also investigated. Cells were treated with 10, 50 and 100 μM CORM-F3 in the presence or absence of 1 μg/ml LPS for 24 h. The medium was collected and used for both a Griess assay and LDH, while the cells were used for the Alamar blue assay.

7.3.5 Alamar Blue Cell Viability Assay

Cell viability was determined using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK). The assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised (blue) form to a reduced (red) form. The intensity of

the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control.

7.3.6 Lactate Dehydrogenase Assay

The effect of CO-RMs on cell viability was also assessed using the lactate dehydrogenase (LDH) assay. LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH based cytotoxicity detection kit (Roche Diagnostics, UK) is a method for the colourimetric assay for the quantification of cell death and cell lysis based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The assay was carried out according to the manufacturer's instructions.

7.3.7 Assay for Nitrite Levels

Nitrite levels were determined using the Griess method. Briefly, the medium from treated cells cultured in 24 well plates was removed and placed into a 96 well plate in triplicate (50 µl per well). To generate a standard curve, 50 µl of sodium nitrite (0 µM to 300 µM in medium) was added to the plate in triplicate. Griess reagent was added to each well to begin the reaction. The plate was shaken for 10 min to allow the reagents to mix and then the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The nitrite level in each sample was calculated from the standard curve.

7.3.8 Aortic rings preparation

Transverse ring sections of aortas were obtained from male adult Sprague–Dawley rats (350 g) and suspended under 2 g tension in oxygenated Krebs–Henseleit buffer as previously described (Sammut et al. 1998; Clark et al. 2003). To establish the potential vasorelaxant effects of CORM-F3, aortic rings were precontracted with phenylephrine (1 μM) before addition of CORM-F3 (100 μM).

7.3.9 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$.

7.4 Results

7.4.1 CO release and related toxicity of CORM-F compounds

Immediately evident is the varying degrees of CO release from each compound. CORM-F3 is the more potent CO releaser (Figure 7.4A and B) generating 18 μM of MbCO. CORM-F8, in which the bromine is replaced with a chlorine, is still capable of releasing CO but generates only a third of the MbCO as CORM-F3 in the same time (Figure 7.6A and B). The release of CO from CORM-F3 and CORM-F8 is slow and steady, neither releasing an equilivant amount of CO as the concentration of CO-RM added. Both CORM-F7 and CORM-F11 do not release any CO (Figure 7.5A and B; Figure 7.7A and B). CORM-F7 is analogous to CORM-F8 with the exception of a methyl side chain, stressing the importance of the methyl group. While, CORM-F11 contains two methyl groups and no halogen group, in this instance demonstrating it is the combination of methyl and halogen group that promote CO release. CORM-F3 vs. CORM-F8 also demonstrates the halogen group can also determine the CO release.

None of these compounds show any significant reduction in metabolism at 10-100 μM as demonstrated by the alamar blue assay. But they do all significantly reduce metabolism at 200 μM (Figure 7.4D, Figure 7.5D, Figure 7.6D, Figure 7.7D). Interestingly, CORM-F7 and CORM-F8 demonstrate an increase in LDH release (indicating toxicity) starting at 100 μM (Figure 7.5C, Figure 7.6C), while, CORM-F11 shows damage at 10 μM (Figure 7.7C). This could indicate the CO-RMs are causing some damage to the cell membrane prior to affecting the functioning of the cell (as determined by metabolism). CORM-F3 on the other hand is only toxic at 200 μM (Figure 7.4C), making it the least toxic of the four.

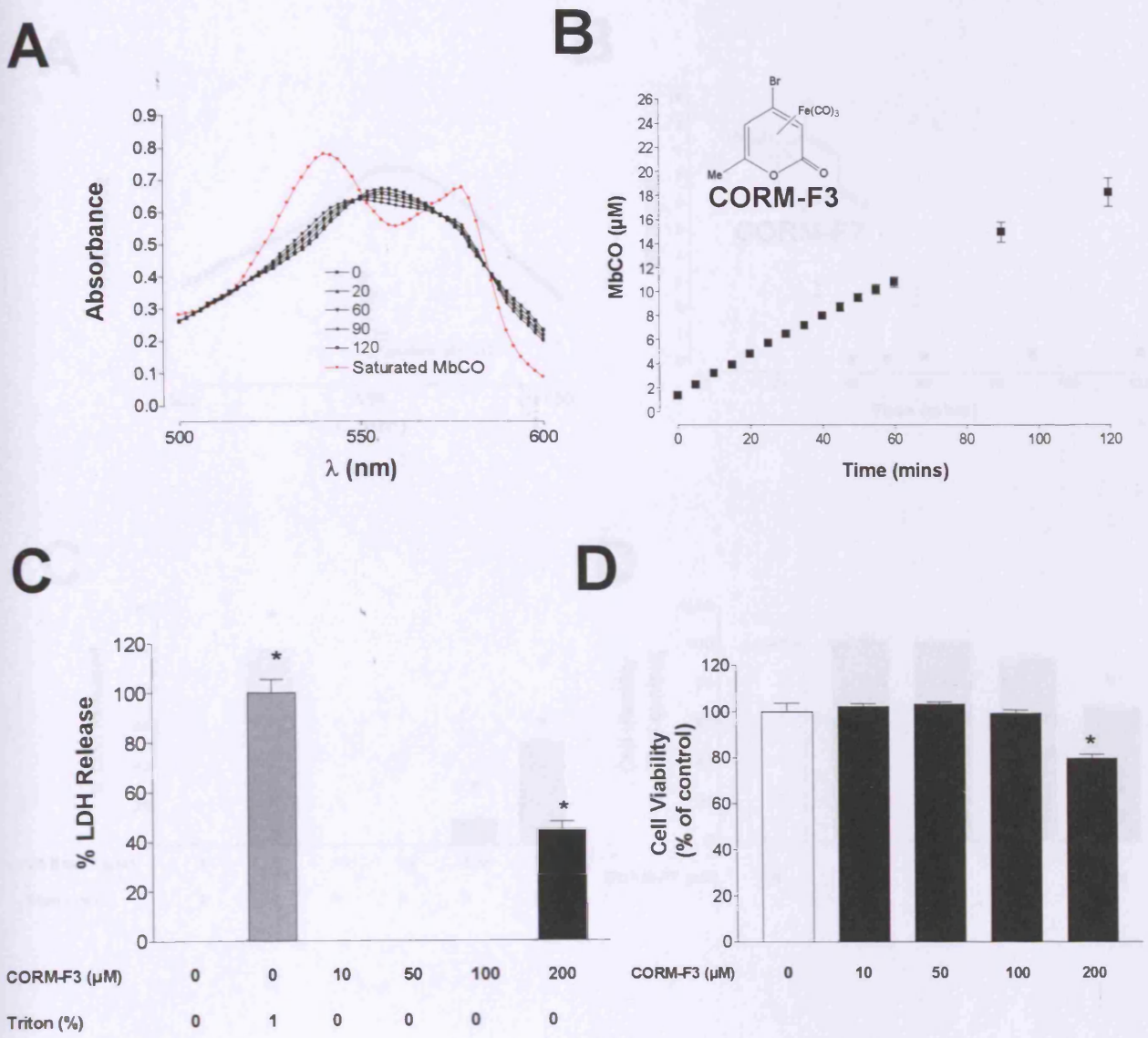


Figure 7.4:- CORM-F3; CO release and effect on cell viability in macrophages.

(A) CORM-F3 (40 μM) was added to a 66 μM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-F3 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb and sample readings at 540 nm. This is the amount of CO released in solution. Cell viability was assessed 24 h after exposure of macrophages to CORM-F3 (10-200 μM) using (C) the LDH assay or (D) Alamar Blue. Viability was expressed as percentage of maximal damage (triton) and minmal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

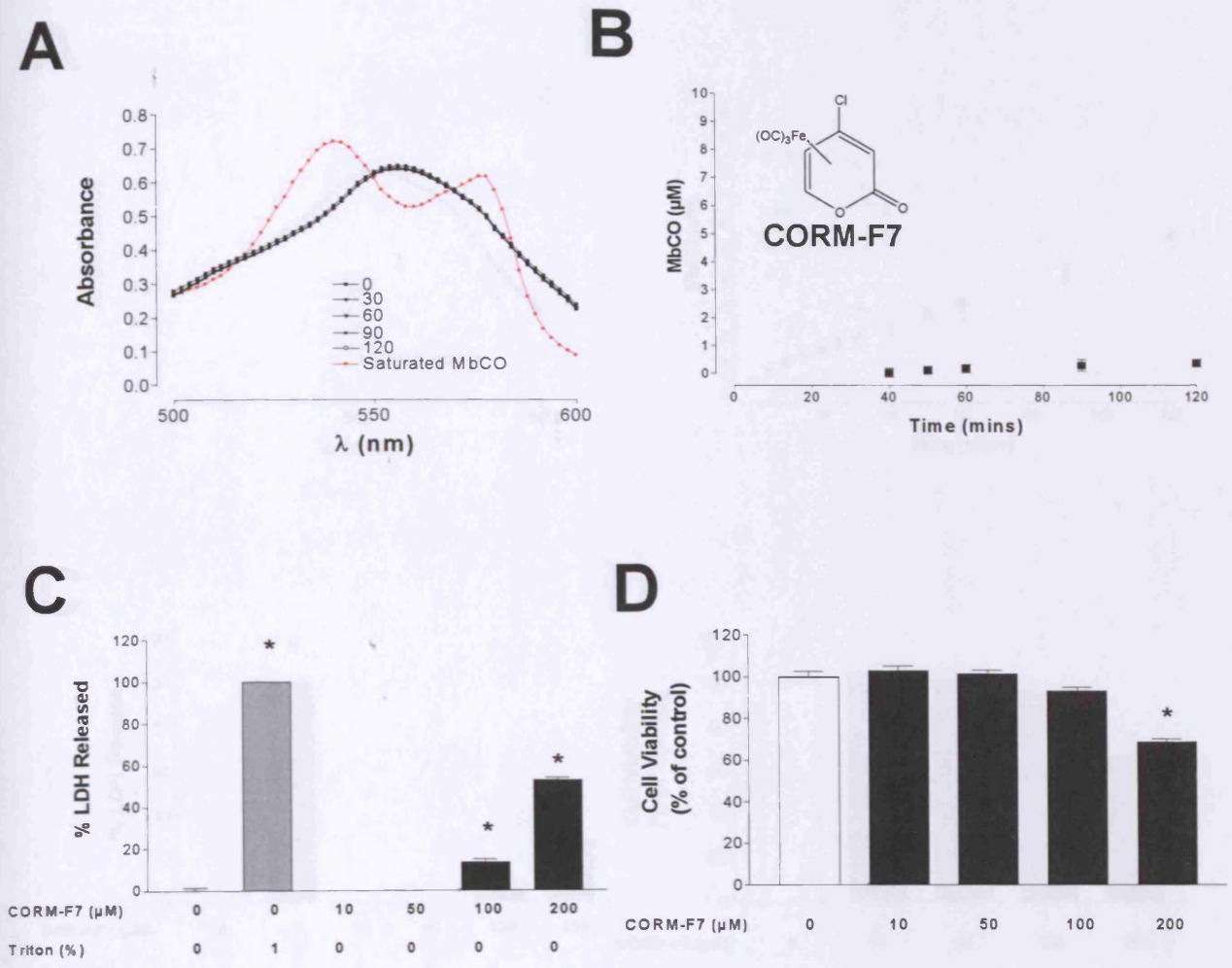


Figure 7.5:- CORM-F7; CO release and effect on cell viability in macrophages.

(A) CORM-F7 (40 μM) was added to a 66 μM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-F7 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb and sample readings at 540 nm. This is the amount of CO released in solution. Cell viability was assessed 24 h after exposure of macrophages to CORM-F7 (10-200 μM) using (C) the LDH assay or (D) Alamar Blue. Viability was expressed as percentage of maximal damage (triton) and minmal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

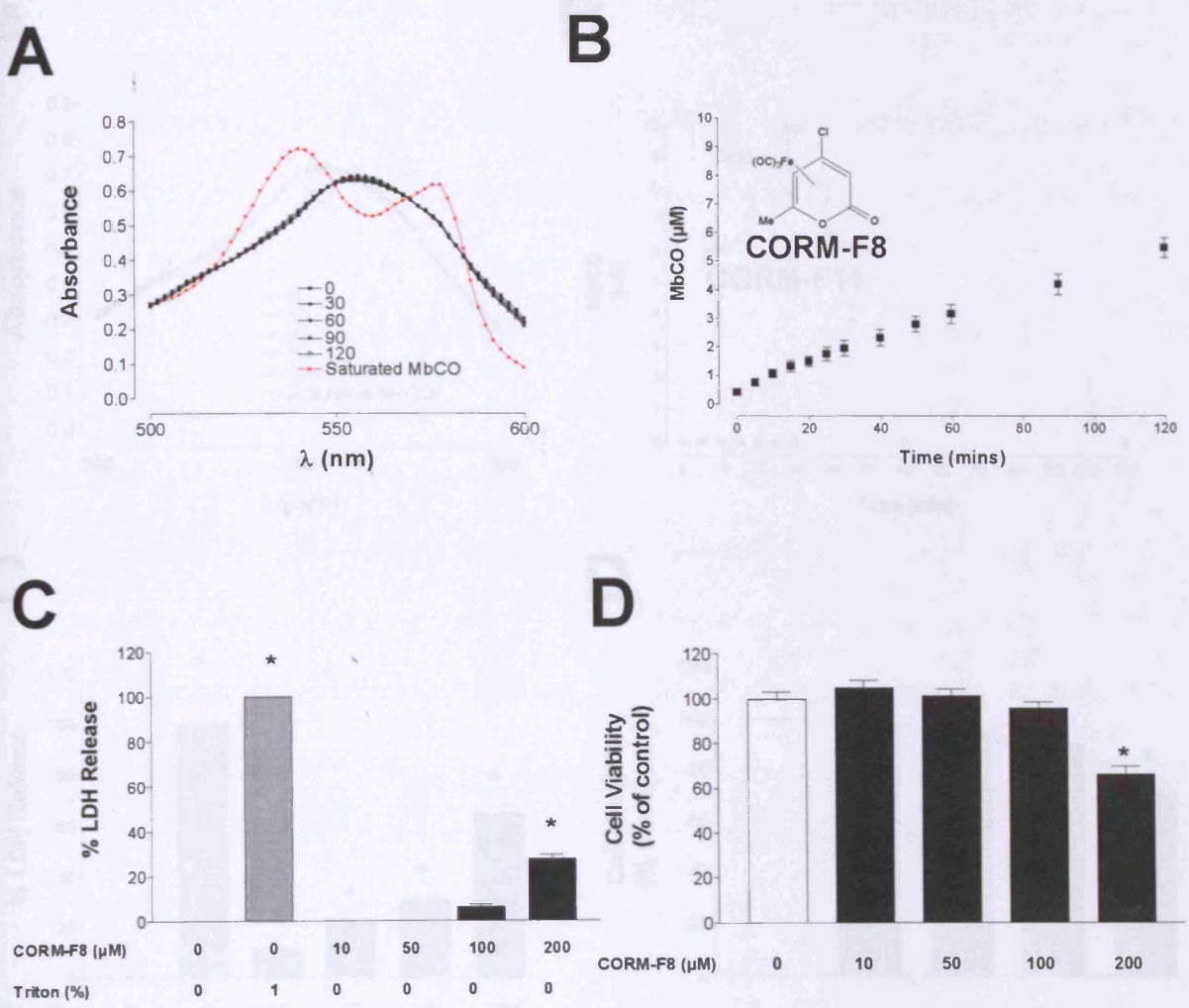


Figure 7.6:- CORM-F8; CO release and effect on cell viability in macrophages.

(A) CORM-F8 (40 μM) was added to a 66 μM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-F8 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb and sample readings at 540 nm. This is the amount of CO released in solution. Cell viability was assessed 24 h after exposure of macrophages to CORM-F8 (10-200 μM) using (C) the LDH assay or (D) Alamar Blue. Viability was expressed as percentage of maximal damage (triton) and minmal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

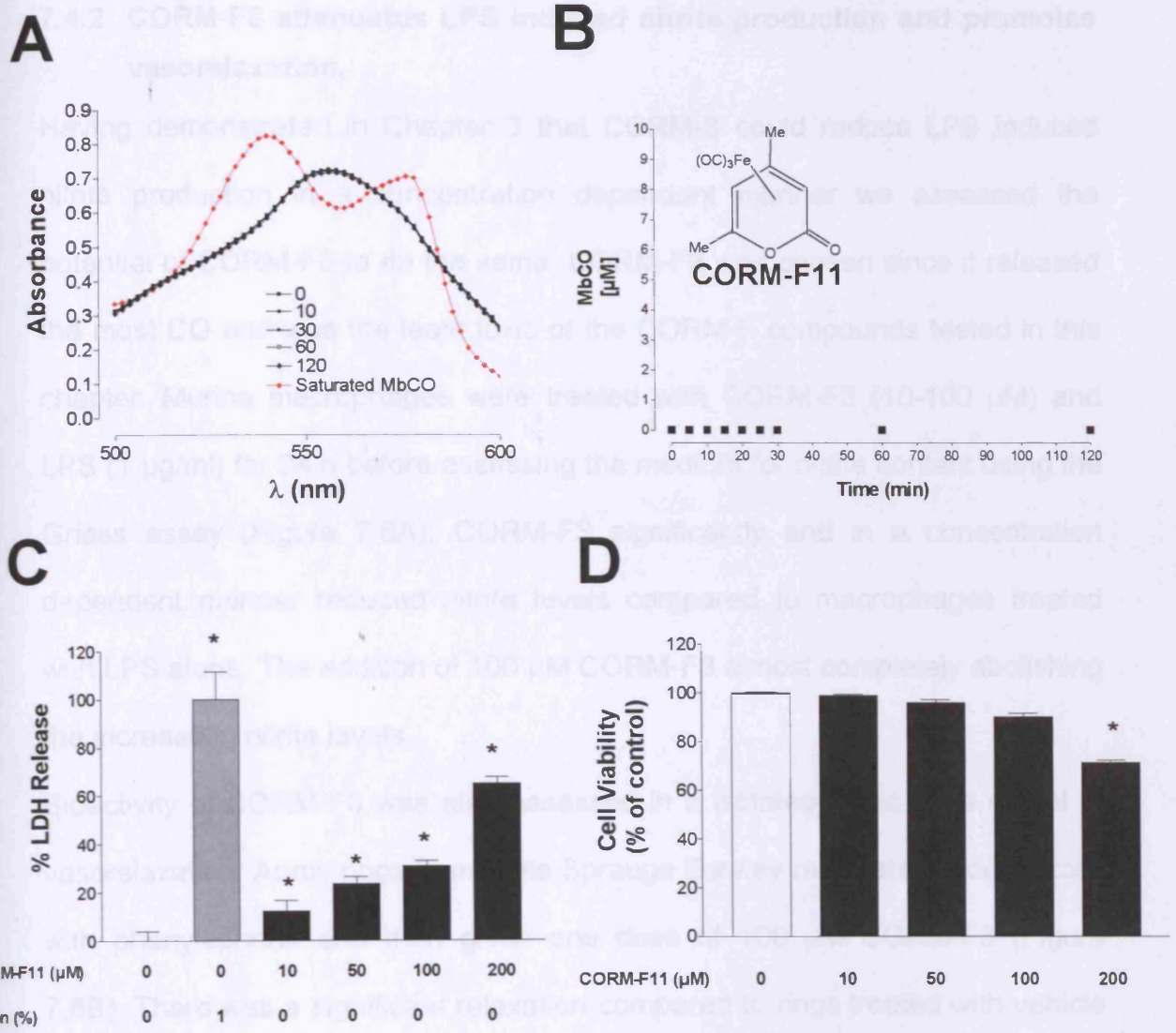


Figure 7.7:- CORM-F11; CO release and effect on cell viability in macrophages.

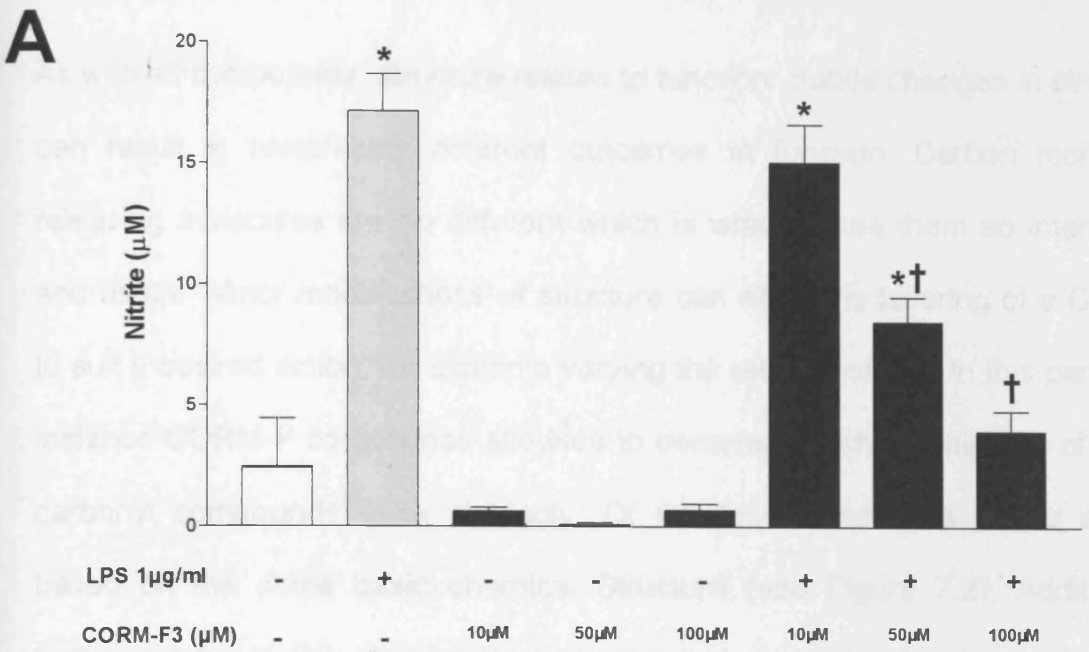
(A) CORM-F11 (40 μM) was added to a 66 μM myoglobin solution. The conversion of myoglobin to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH = 7.4. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-F11 to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively. Myoglobin was saturated (MbCO) by bubbling deoxyMb with CO for 5 min. (B) The MbCO concentration was derived from the increase in absorption between deoxyMb and sample readings at 540 nm. This is the amount of CO released in solution. Cell viability was assessed 24 h after exposure of macrophages to CORM-F11 (10-200 μM) using (C) the LDH assay or (D) Alamar Blue. Viability was expressed as percentage of maximal damage (triton) and minmal damage (control). Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control.

7.4.2 CORM-F3 attenuates LPS induced nitrite production and promotes vasorelaxation.

Having demonstrated in Chapter 3 that CORM-3 could reduce LPS induced nitrite production in a concentration dependent manner we assessed the potential of CORM-F3 to do the same. CORM-F3 was chosen since it released the most CO and was the least toxic of the CORM-F compounds tested in this chapter. Murine macrophages were treated with CORM-F3 (10-100 μM) and LPS (1 μg/ml) for 24 h before assessing the medium for nitrite content using the Griess assay (Figure 7.8A). CORM-F3 significantly and in a concentration dependent manner reduced nitrite levels compared to macrophages treated with LPS alone. The addition of 100 μM CORM-F3 almost completely abolishing the increase in nitrite levels.

Bioactivity of CORM-F3 was also assessed in a isolated aortic rings model of vasorelaxation. Aortic rings from male Sprauge Dawley rats were precontracted with phenylephrine and then given one dose of 100 μM CORM-F3 (Figure 7.8B). There was a significant relaxation compared to rings treated with vehicle alone.

Obviously without further investigation we can only infer that the bioactivity whitnessed with CORM-F3 is as a direct result of the CO released and not the compound itself or a product of its metabolism. But we can be sure the compound is biologically active and worthy of such further investigation.



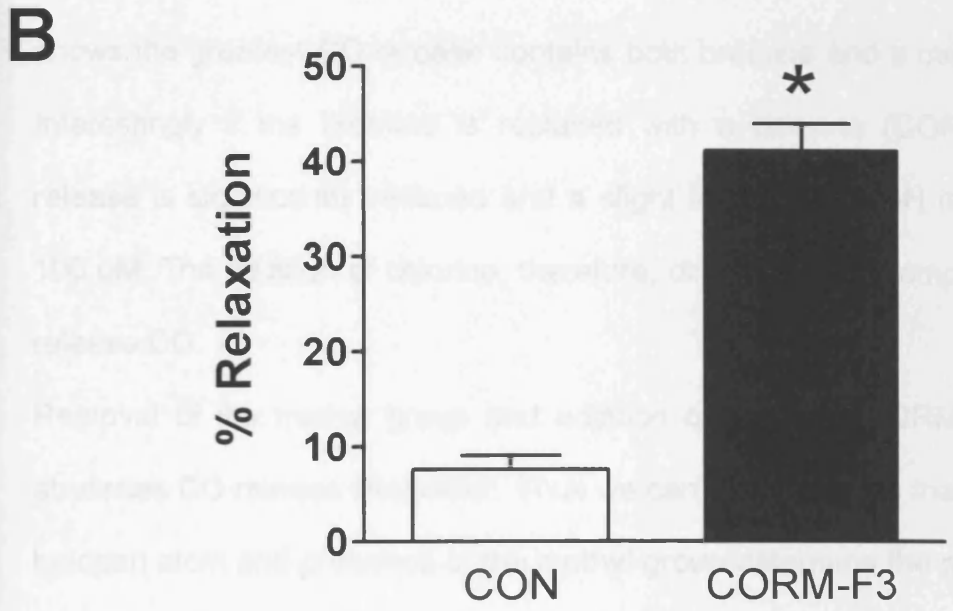


Figure 7.8:- CORM-F3 ellicits both vasorelaxation and a reduction in LPS stimulated nitrite production.
(A) RAW264.7 macrophages were exposed to 1 μg/ml LPS in the presence or absence of CORM-F3 (10-100 μM) and nitrite production was assessed at 24 h. Control cells were incubated with medium alone. (B) Transverse ring sections of aortas were obtained from male adult Sprague–Dawley rats (350 g) and suspended under 2 g tension in oxygenated Krebs–Henseleit buffer. To establish the potential vasorelaxant effects of CORM-F3, aortic rings were precontracted with phenylephrine (1 μM) before addition of CORM-F3 (100 μM).

7.5 Discussion

As with all compounds, structure relates to function. Subtle changes in structure can result in remarkably different outcomes in function. Carbon monoxide releasing molecules are no different which is what makes them so interesting and useful. Minor modifications of structure can allow the tailoring of a CO-RM to suit a desired action, for example varying the release of CO. In this particular instance CORM-F compounds allow us to demonstrate the multiplicity of metal carbonyl compounds quite strikingly. Of the four compounds tested all are based on the same basic chemical Structure (see Figure 7.2). Addition of further groups to this starting structure result in a variety of outcomes that go some way to explaining how to improve and shape function. CORM-F3 which shows the greatest CO release contains both bromine and a methyl side chain. Interestingly if the bromine is replaced with a chlorine (CORM-F8) the CO release is significantly reduced and a slight increase in LDH is observed with 100 μM. The addition of chlorine, therefore, diminishes the compounds ability to release CO.

Removal of the methyl group and addition of chlorine (CORM-F7) practically abolishes CO release altogether. Thus we can infer from this that both the exact halogen atom and presence of the methyl group determine the release of CO to a great extent in these compounds. But both are required to achieve a significant CO release. Important to note is that altering the halogen group and/or adding/removing the methyl group does not significantly affect cell toxicity. Interestingly, cells show more damage to the cell membrane than a reduction in metabolism suggesting that long term CO-RM exposure may present more of an issue on toxicity than short term. CORM-F11 demonstrates

that by removing the halogen group and replacing with a further methyl group both CO release and toxicity are greatly affected. CORM-F11 releases no CO and has an effect on LDH at only 10 μM. The function of the compound entirely altered compared to CORM-F3. As in Chapter 4 the most important issues to stress are the shear potential of CO-RMs. Almost limitless combinations results in a plethora of potential activities. We have tested well over 70 CO-RMs and more are being generated all the time.

It is intriguing to note that both a halogen and methyl substituent are required for good CO release. The observation parallels the previous reported solution behaviour of these complexes. The substituents could play some role in facilitating 2-pyrone dissociation from the metal centre by sterically and electronically perturbing otherwise favourable $d\pi$-π^* synergic effects seen in the parent 2-pyrone complex. Alternatively, an increase in the strain in the carbonyl fragment of the 2-pyrone iron tricarbonyl complexes could favour ring-opening and further reactions.

7.6 Conclusion

The development of new compounds capable of releasing CO in a controlled fashion is fast accelerating. Ever since CORM-3 was first synthesised the potential of metal carbonyl compounds has been realised and CO-RMs are being synthesised to build up a large portfolio of candidates for future studies. CORM-F compounds demonstrate the ability to subtly manipulate structure to achieve variation in function. The potential number of CO-RMs is therefore almost limitless. Characterising the many compounds is vital to identify CO-RMs

that may have specific roles, which can be exploited for certain pathophysiological conditions.

8 General Discussion

8.1 Analysis of Methodology

This Thesis has been structured according to the topic of each chapter rather than by the methodology used. The chapters therefore are not presented in chronological order of completion rather, upon completion of the whole work they have been arranged to best introduce each aspect of the project.

8.1.1 Cell Culture Methodology

Cell culture is often used for studying the specific effects of environmental changes or chemical mediators on particular cell types. The macrophage cell line used for this study was derived from a murine source, while endothelial cells were from bovine aortic tissue. Visually the cells maintained their respective morphologies but it would be of further benefit to compare them to primary cells in respect of their HO activity, HO-1 expression and further biological activities. However, the data obtained is supported by a number of other reports using both macrophages and endothelial cells from other mammalian species (Motterlini, Foresti, Intaglietta, & Winslow 1995a; Foresti et al. 2000; Clark et al. 2000b; Balogun et al. 2003; Sawle et al. 2005; Nakamichi et al. 2005; Tsoumakidou et al. 2005; Ramirez-Emiliano, Gonzalez-Hernandez, & Arias-Negrete 2005; Paoliello-Paschoalato, Oliveira, & Cunha 2005). The cells lines were passaged prior to confluence (~80% which was determined visually) and maintained in both cases until passage 30 at which point cells were discarded and a new cell line aquired. Cells were maintained in a sufficient

volume of culture medium at all times and kept in an incubator at 37 °C with an atmosphere of 5% CO_2 balanced with air.

8.1.2 Biochemical and Molecular Biology Techniques

The major biochemical and molecular biology techniques used were; (i) the HO assay, (ii) the nitrite assay, (iii) the myoglobin assay, (iv) cell viability assays and; (v) Western blot assay for HO-1 and iNOS protein expression.

The haem oxygenase assay was first described by Tenhunen *et al* in 1970 and has been used extensively by our laborartory to measure intracellular and tissue HO activity (Tenhunen, Marver, & Schmid 1969; Motterlini et al. 1995b; Motterlini, Hidalgo, Sammut, Shah, Mohammed, Srai, & Green 1996). The assay used in this Thesis reflects the relative HO activity in the sample as "pmol. bilirubin/mg prot. /h". Although the value obtained is not directly comparable to the amount of bilirubin measured in the culture medium after stimulation of the HO pathway, the fact that the substrate (hemin) and the cofactors NADPH, glucose-6-phospahte and glucose-6-phosphate-1-dehydrogenase and biliverdin reductase were all added in excess and the procedure was carried out under controlled conditions must be considered when interpreting the data.

The Griess method for the measurement of nitrite is a tried and tested protocol for assessing the nitrite content of cell culture supernatant. Sample nitrite is determined by comparison of the absorbance of treated cell supernatant to a standard of known nitrite concentrations which is run each assay. In this Thesis

the assay is used to infer the anti-inflammatory effect of CO-RMs. Nitrite, however, is only an indication of iNOS activity and much debate still centres on the true product of iNOS activity. Measurement of both nitrate and nitrite can give a better overall picture of the true effect, requiring the conversion of nitrate to nitrite, but in this instance measurement of nitrate proved unreliable.

The myoglobin assay is based on the unique property of myoglobin to exist as various spectra depending on whether it is deoxygenated or carboxylated. This allows us to measure CO release from CO-RMs. Myoglobin has a high affinity for CO and as such, quickly mops up any CO in the solution irreversibly (for the sake of this assay) converting it to carboxymyoglobin with its associated pinky hue. As mentioned in Chapter 2 a mathematical iteration is required to determine the CO release of CO-RMs assuming deoxymyoglobin is 0 μM and myoglobin bubbled with CO is 100% saturated. The assay has proved to be a reliable method for measuring CO with total CO release and kinetics of CO-RMs being mirrored in tests using a CO electrode. There are alternative techniques described in the literature which can also be used to determine CO concentration. These techniques allow for the quantification of CO levels in cell culture supernatant and as such are not directly applicable to measuring the CO release from CO-RMs. One technique in particular developed by Stonek *et al*, an optimised nonradioactive spectrophotometrical detection of dissolved CO at physiological concentrations with a detection limit of less than 50 μg CO/l tissue culture supernatant, is a relatively simple assay to detect CO release from cultured cells (Stonek et al. 2004). The application of this as a direct method to test CO release from CO-RMs is not practical since it relies on the same

principal as the myoglobin assay (substituting haemoglobin for myoglobin) but is far more complicated and time consuming than the simple addition of CO-RMs directly to deoxy-Mb. This technique, however, could be used to determine the extent of CO release by CO-RMs in cell culture treatments and whether the CO release detected in the myoglobin/CO electrode assays is comparable to that of the CO release *in vitro*.

Cell viability is often a contenscious issue especially when using only one of the numerous methods available. Often what is measured is a factor which infers the overall health of the cells rather than a definitive measurement of viability. In this Thesis it was decided that the best means to overcome this problem was to use various methods to determine cell viability and compare the results of each to give an overall viability. The Alamar blue assay measures cell metabolism. On its own it is a poor indicator of cell viability as metabolism can be affected by a large number of factors including confluence of the cells and the treatment itself. The assay, however, is quick, simple and inexpensive. The measurement of LDH in the supernatant of treated cells was also measured. This gives an indication of the state of the cell membrane integrity. A large release of LDH indicates the cell membrane has been damaged since LDH is an integral part of the cell membrane, which infers a loss of viability. LDH content, however, can also be affected by confluence and the treatment apllied. The assay is quick and simple, and leaves the cells to be used for subsequent assay as only the supernatant is required. The final indicator of cell health used in this Thesis was the Trypan blue exclusion assay. Cells that have had their cell membrane breached allow the uptake of the Trypan blue dye, while intact cells do not.

Hence, the percentage of stained cells to unstained cells can be calculated using a microscope and the viability determined. A breached cell membrane indicates a loss of viability but does not exclude apoptotic cell death where the membrane blebs rather than splits. The assay is inexpensive, simple but time consuming and not wholly consistent. Each assay, therefore, has limitations in directly determining cell viability but the combination of all three can allow us to be more precise in the labelling of compounds as either toxic or non-toxic.

Western blot is an established molecular biology technique for measuring relative protein expression in cells and tissues (Laemmli 1970); therefore, every assay was run with both negative and positive controls (untreated cells and recombinant HO-1 protein or LPS stimulated cells in the case of HO-1 and iNOS respectively). Since Western blot analysis of HO-1 and iNOS expression is only used in conjunction with HO-activity and nitrite concentration, the actual amount (µg; µmoles etc.) of HO-1/iNOS protein are not necessary; each experiment is presented by a representative blot which has been scanned in to a microcomputer, cropped to the right size and printed.

8.2 Hypothesis and Aims

The hypothesis behind the work carried out for this Thesis is:

CO-RMs, through the action of CO, can mimick the action of endogenously genereated CO in a murine model of inflammation.

The data presented in this Thesis are consistent with the hypotheis, although the mechanisms behind the exact workings of CO-RMs have not been completely explored. However, this will be addressed in the next section (Paragraph 8.3, 8.4 and 8.5).

The Aims of this Thesis were stated at the close of the introduction and were;

Test the prototypic water soluble CO-RM (CORM-3) to determine its toxicity in various cell lines, its effect on the HO system and its regulation of the inflammatory response inferred from TNF-α, nitrite and iNOS levels in macrophages.

In Chapter 3 we demonstrated that CORM-3 was capable of CO release in the myoglobin assay and that incubating the compound in biological buffer overnight inactivated it preventing further CO release.

Due to the overwhelming evidence for a role for CO in the resolution of inflammation we decided that we would investigate the effects of CORM-3 on LPS induced nitrite production which is generally regarded as a way of inferring inflammation (SCHAUS 1956; Ohta et al. 2003; Sawle, Foresti, Mann, Johnson, Green, & Motterlini 2005). CORM-3 and not iCORM-3 potently reduced nitrite

production which was shown to be independent of HO activity and HO-1 expression despite the ability of CORM-3 to increase both at certain concentrations. The increase in HO activity and HO-1 expression seemed to be reliant on a change in the redox status of the cell as NAC was able to completely inhibit both, whilst, gluthathione levels were also shown to be reduced at CO-RM concentrations above 50 μM. Toxicity was not an issue with CORM-3 found not to effect LDH, Alamar blue and Trypan blue with concentrations up to 500 μM.

CO is known to affect haem containing compounds and has been shown to act on guanyl cyclase in a similar fashion to NO. The exact mechanism for reducing nitrite in this case is independent of the redox induction of the HO pathway, unrelated to the compound (ineffectiveness of iCORM-3) and is not as a result of increased iNOS expression. This would suggest CORM-3 acts to reduce the activity of the iNOS enzyme. Indeed, CO has been suggested to bind iNOS and cause a conformationl distortion of the active site (White & Marletta 1992; McMillan, Bredt, Hirsch, Snyder, Clark, & Masters 1992). As well as nitrite the effect of CORM-3 on TNF-α, a more direct indicator of inflammation was investigated. CORM-3 was able to significantly attenuate LPS induced TNF-α production, again indicating the potent anti-inflammatory actions of this compound.

Investigate the ability of newly synthesised CO-RMs to release CO using the myoglobin assay to determine new CO-RMs with the potential to forward the study.

Having established that metal carbonyl compounds can be manipulated to serve a biological action, in Chapter 3 we broadened the investigation into these intriguing molecules. New CO-RMs were based on metals commonly found in biological systems such as iron and manganese. The early ruthenium compounds, although extremely easy to manipulate from a chemical point of view could in theory cause some problems with biocompatibility especially if used in clinical treatments for a long period of time. This is not to say they are toxic since ruthenium compounds are already in use in the clinical setting of cancer therapy (Alessio, Iengo, Zorzet, Bergamo, Coluccia, Boccarelli, & Sava 2000; Alessio, Mestroni, Bergamo, & Sava 2004a; Alessio, Mestroni, Bergamo, & Sava 2004b). But CO-RMs based on metals present in biology are, perceptively, more of an attraction. Both iron and manganese are two common metals present in enzymes and protein structures and form a crucial core in their function and activity. With the help of Professor Brian Mann (University of Sheffield) and Ian Fairlamb (University of York) further CO-RMs were developed and tested in Chapter 4 to allow us to select the most promising to take forward.

Having established the myoglobin assay as a reliable method for determining CO release we started to interpret a vast portfolio of CO-RMs for their ability to release CO (see appendix 11.1). The different chemistry of the CO-RMs studied dictated a different degree of CO release. Many CO-RMs were found to release little or no CO; however, there were a number of compounds that looked promising: CORM-307, CORM-308, CORM-311, CORM-312 and CORM-319, of which all contained iron as a metal centre except CORM-312. Interestingly, the kinetics of CO release was quite varied demonstrating the potential of CO-

RMs to be refined and tailored to release CO dependent on the requirement. This 'tailoring' is best exemplified in Chapter 7 by the CORM-F series where minor modification to structure drastically alters function, whilst, Chapter 6 explores the characteristics of CORM-A1 an entirely novel non-metal containing CO-RM. By learning from each CO-RM we can forward the development of future new and improved CO carriers for the therapeutic delivery of CO in humans.

Test compounds deemed to be worthy of further study from Chapter 4 to determine their toxicity, effect on the HO system and regulation of the inflammatory response.

Among the CO-RMs that showed a promising release of CO reported in Chapter 4 only CORM-311 and CORM-319 were found to be non-toxic and thus investigated further. Both CO-RMs liberated CO gradually over time in stark contrast to the instantaneous release by CORM-3. The effect of both these compounds was investigated on HO activity and HO-1 expression as well as nitrite levels in LPS induced macrophages in a range of concentrations that have been found to be biologically active from CORM-1. We found that CO-RM-311 and CORM-319 were not toxic, and that both were able to reduce nitrite whilst having no effect on either HO activity or HO-1 expression. The effect of CORM-319 could be associated with its ability to release CO since iCORM-319 was unable to elicit any response. The lack of a proper iCORM-311, however, made it impossible to confirm that CO was involved in its bioactivity. Without further investigation it is speculative to suggest the kinetics of CO release play any role in the actions of CO-RMs in comparison to CORM-3.

Determine the CO releasing profile and bioactive properties of CORM-A1, a newly identified CO generator that does not contain a transition metal.

The rationale for the development of a non-metal contaning CO-RM came as a surprise, albeit a welcome one, which allowed us to investigate and compare a completely novel class of CO-RM with the existing metal carbonyl ones. Without the presence of the transition metal centre such CO-RMs could be less reactive, have a greater solubility in water and have a high biocompatibility. The potential of sodium boranocarbonate (CORM-A1) to release CO had been used chemically to synthesise carbonyl compound but never biologically. The compound itself releases CO in the presence of protons (H^+). The release of CO was found to be dependent on concentration, temperature and pH. In water, the compound is stable for many days, however, it can be inactivated by adding a single drop (10 µl) of 1 M HCL which stimulates a vigorous release of CO. The release of CO, under standard conditions used to test all the previous CO-RMs, demonstrates that CORM-A1 is a gradual CO releaser similar to CORM-319 (half life = 21 min at 37 °C, pH = 7.4). It is unable to attenuate LPS induced nitrite generation in macrophages and was found, unlike CORM-3, not to stimulate HO activity. It had no effect on HO activity in either bovine aortic cells or girardi cells. Its apparent lack of pharmacological activity is in apparent contrast to its effects in the isolated aortic rings, Langendorff and isolated kidney perfusion system where CORM-A1 has been shown to induce vasodilation and offer protection against ischeamia-reperfusion respectively (Heart data unpublished; Kidney data in press; Aortic rings data in (Motterlini, Sawle, Hammad, Bains, Alberto, Foresti, & Green 2005)). The different actions of the various CO-RMs tested could very well depend on how and where they

release CO and the effect of the resultant post CO release compound. It should be noted that CORM-A1 is also quite a relatively good reducing agent.

Demonstrate how structure can affect CO release, toxicity and nitrite using CORM-F compounds.

Having investigated such a large number of CO-RMs Chapter 7 illustrates the process of developing new versions of CO carriers. A number of variations based on a core structure were investigated and it was shown that small alterations in structure could lead to big differences in function. Important for these compounds was the type of halogen group and the presence of a methyl side chain. Both determined the amount of CO released and the observed toxicity. This chapter elegantly demonstrates how many potential structures can be put forward on a set core structure. Having identified the important factors regarding CO release and toxicity further CO-RMs based on this information can then be developed to improve and advance the original, whilst better mimicking the function of CO biologically.

The data from Chapters 3, 5 and 7 support the overall hypothesis. The exact mechanism by which CORM-3, CORM-319 and CORM-F3 achieve this is not yet fully understood although, it is likely through the actions of CO. CORM-311 also was able to mimick the anti-inflammatory action of CO but the lack of a inactive version prevented CO from being directly implicated in its action. Of the other CO-RMs tested none were capable of eliciting a biological effect, including

CORM-A1 which is interesting since it has been shown to be active in a number of other systems.

8.3 Reflections

CO has been an intrinsic part of life on this planet since long before human history began. Recently suggested to have been involved in the synthesis of the first amino acids there is now consistent publication of studies supporting the beneficial effects elicited by CO. Only twenty years ago this would have been almost unthinkable. Long regarded as a poisonous gas the once considered 'waste' product of HO activity is finding favour amongst the scientific community eager to elucidate more about this simple diatomic molecule. CO derived from HO activity acts as a signalling molecule with anti-hypertensive, anti-proliferative and anti-apoptotic properties (Morita, Perrella, Lee, & Kourembanas 1995; Durante & Schafer 1998; Johnson, Kozma, & Colombari 1999; Brouard, Otterbein, Anrather, Tobiasch, Bach, Choi, & Soares 2000; Morse & Sethi 2002). Other physiological roles of CO described to date include prolonged survival of transplanted organs, suppression of atherosclerotic lesions, prevention of reperfusion-induced ventricular fibrillation in the myocardium, and protection against ischemic lung injury (Otterbein, Mantell, & Choi 1999; Luckraz, Tsui, Parameshwar, Wallwork, & Large 2001; Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003a; Nakao et al. 2003a). Not to mention the ability of CO at low concentrations to modulate the production of inflammatory mediators by decreasing pro-inflammatory cytokines such as TNF-α and increasing crucial anti-inflammatory molecules such as IL-10 in various *in vitro* and *in vivo* experimental models (Tamion et al.

1999; Sethi, Otterbein, & Choi 2002a; Wagener et al. 2003b; Sawle, Foresti, Mann, Johnson, Green, & Motterlini 2005).

It was only just over 10 years ago not long after the discovery of NO as the EDRF, that the first scientific groups began to investigate and propose that CO may not just be a by-product of HO activity and that it may function instead in a fashion similar to NO (Marks, Brien, Nakatsu, & McLaughlin 1991; Dawson & Snyder 1994; Anfossi et al. 2001). Since those first publications, the study of CO has intensified and as with all things it has been shown that it is concentration which determines if it is toxic or a cure. The development of NO-releasing compounds has made dramatic improvements in the way in which we can investigate the role of NO in biological systems and has opened up new stratergies in the treatment of chronic cardiovascular disease in the clinic (Napoli & Ignarro 2003). Research in to the CO field has been hampered by a lack of an equilivant group of compounds. The question was and still is; how to emulate the action of CO? i) CO gas? Too non-specific and unfeasibly complicated to administer to humans, not to mention potential long-term health problems. Most of the work to date though has been carried out using CO gas as a substitute for endogenous CO, although, not perfect the use of CO gas has facilitated the elucidation of many now well known aspects of CO function. Important in the discussion of this Thesis is the work by Otterbein *et al* demonstrating the anti-inflammatory effects of CO and that these effects were mediated through p38MAPK (Otterbein et al. 2000b). The use of CO gas by White and Marletta also helped to demonstrate that CO can potently inhibit the conversion of l-arginine to citrulline and NO by nNOS and macrophage NOS (White & Marletta 1992). Not to mention observations made by Sethi *et al* that

CO gas could inhibit the expression of LPS-induced TNF-α production *in vitro* (Sethi, Otterbein, & Choi 2002b). In fact CO gas has been vitally important in understanding the effects of CO in inflammation. It is, therefore, intriguing to see whether the use of CO-RMs, which generate a more physiological relevant CO release, agree or dispute the current findings with CO gas. CO gas has also been used to demonstrate a role in inhibition of platelet aggregation, sGC activation, transplant survival, protection against ischaemia/repurfusion injury and other important physiological processes which are highlighted in the introduction (paragraph 1.11.3). ii) Methylene chloride and other pro-drugs? Again non-specific, highly carcinogenic and require good liver function. iii) Which leaves us with increasing HO activity to generate CO as a by-product. There is potential here but most ways of increasing HO in a living person are impractical, are non-specific for only HO activity, are likely only to affect an acute response and also involves the generation of various other compounds; biliverdin; bilirubin; and Fe with subsequent ferritin synthesis. As such, attributing any observed effects in such a model specifically to CO is not easy.

The discovery that transition metal carbonyls used for the purification of metals could release CO in a myoglobin assay determined by the resultant convertion of myoglobin to MbCO offered a potential novel solution to harnessing the very real protective power of CO in a convenient, safe and specific manner (Motterlini, Clark, Foresti, Sarathchandra, Mann, & Green 2002b; Clark, Naughton, Shurey, Green, Johnson, Mann, Foresti, & Motterlini 2003a). These compounds termed CO-RMs have been investigated and developed by our group and collaborators as an experimental tool for the elucidation of the role of CO in the body with the long term goal to develop therapeutic agents. Early CO-

RMs allowed us to determine the potential of manipulating the compounds to suit biological compatibility. Preliminary experiments with the ruthenium and manganese carbonyl complexes (CORM-2 and CORM-1 respectively), soluble only in organic solvents, revealed they could be used safely at low concentrations to promote blood vessel relaxation and mitigate hypertension *in vivo*. Since those initial experiments the bioactivity of CORM-1 and CORM-2 has been confirmed by a number of other laboratories in experimental models that have been used to implicate CO as an important contributor to cerebral vasodilatation, suppression of SMC proliferation, angiogenesis, inhibition of apoptosis, improved renal function, anti-inflammatory and immunological responses (Fiumana et al. 2003; Lee, Tsai, & Chau 2003; Jozkowicz et al. 2003; Guo et al. 2004; Koneru & Leffler 2004; Chatterjee 2004a).

The success of these early CO-RMs was such that the development of newer potentially more biologically compatible CO-RMs was a necessity for steering the studies towards a possible pharmaceutical development based on CO delivery to mammals. A collaboration between Dr Roberto Motterlini and Professor Brian Mann (University of Sheffield) accelerated the development of the first prototypic water soluble transition metal carbonyl (CORM-3). As a ruthenium based compound, CORM-3, demonstrated the potential of the carbonyl complexes to release CO and be biologically active. By adding a biological ligand, in the form of an amino acid (glycine), the molecule was made water soluble. CORM-3, released CO instantaneously when added to buffer in a 1:1 ratio with that of the concentration of CORM-3 added. Not toxic in macrophages at concentrations up to 500 μM as shown by the combination of Alamar Blue, Trypan Blue and LDH assays, CORM-3 was the prototypic water

soluble, biologically compatible CO-RM. Similarly ligand modification could also be used in the future to manipulate CO release. The diversity of compounds, therefore, that could be synthesised was almost endless.

With the discovery of CORM-A1 a non-metal containing CO-RM there now exists a new generation of CO carriers that can be compared and evaluated for their potential therapeutic activity. Such diversity in the number of compounds that can release CO in a biological system offers great potential in attaining the goal of a compound that can be used in the clinical setting.

8.4 Future Directions

The overall end goal of CO-RM research and development is the creation of a therapeutic agent capable of carrying and delivering CO in the right concentration to a specific target in the body whereupon it acts to counteract a pathophysiological event. There are many short terms goals to be achieved prior to this and many ways for furthering this study which include:

1) The CO-releasing capabilities and bioactivities of many CO-RMs have to be investigated to allow the refinement and creation of new formulations that are more biological compatible.
2) Investigations should be carried out on the effect of CO-RMs on additional markers of inflammation. These should include interleukins IL-1 and IL-6, two pro-inflammatory cytokines and IL-10 an anti-inflammatory cytokine. The effect on adhesion molecules and platelet aggregation should also be investigated since these factors also contribute to the inflammatory response and have been shown to be

affected by CO. Simple investigation using ELISA is possible in this instance.

3) Test CO gas as a direct comparison with CO-RMs. There are numerous studies which have already produced convincing data as to the effect of CO in inflammation (and many other models) but it would be of benefit to replicate this research in relation to this project in order to confirm the effect in our models.
4) Using the method developed by Stonek *et al* mentioned earlier to test the CO levels in cell culture supernatant after administration of CO-RMs. This would help us to determine the exact level of CO release *in vitro* which we can only infer from the data collected using the myoglobin assay. The more complex environment of cells in culture may accelerate or even hinder the release of CO by CO-RMs which is of crucial importance when reviewing the data.
5) Investigations should be carried out using the p38MAPK inhibitor SB203580. p38MAPK has been implicated in the effect of CO on inflammation, as such inhibition of this pathway would allow us to determine if CO-RMs mediate their effect through the same pathway as CO gas and other methods of simulating CO. This may help to elucidate the different effects on nitrite observed with CORM-3 and CORM-A1.
6) Further research clarifying the exact mechanisms by which CO released from CO-RMs acts in other models is also required. The chemical pathways involved such as NF-κB or cGMP and the identification of new targets for CO (mitochondria and NADPH oxidase) may help to establish key components in the activity of CO-RMs.

Current investigations into CO-RMs have studied it in particular models and systems (i.e. murine macrophage model of inflammation). These early studies allow us to define the biological suitability, toxicity, concentration range of use, mechanism of action and the overall therapeutic potential. Further studies into wider ranging aspects of CO function are required. The role of CO has been investigated in many models and its effect on various biological functions has been reported. CO-RMs should be investigated in as many such systems as possible to corroborate or dispute the existing findings of CO gas and HO upregulation based experiments. Having established *in vitro* and *ex vivo* actions of CO, major research should be conducted into the *in vivo* effects of CO-RMs that have been proven to function well, elicit no obvious toxicity in any model they have been tested in and provide a desired therapeutic action.

Of particular interest is the identification of sodium boranocarbonate ($Na[H_3BCO_2]$ (CORM-A1), a non-metal containing agent. Its apparent lack of biological activity despite a CO release similar to that of both CORM-319 and CORM-311, two active CO-RMs, raises a number of important issues; (i) is CO responsible for the effects observed or is it due to the presence of a metal? and (ii) where precisely are the CO-RMs acting? These questions must be addressed considering the success of CORM-A1 in other models.

In regards to this study an end goal should be to attenuate inflammation in an *in vivo* model using CO-RMs, thus, proving its function in an actual biological system.

8.5 Overall Conclusion

CO-RMs offer a great opportunity for the development of a novel class of therapeutic agents based around the action of CO, which has been considered a poison for a long time. CORM-3, a prototypic water soluble CO-RM, already has a number of desirable characteristics which can be improved upon for the refinement and development of future transition metal carbonyls. Here, we report that CORM-3 is capable of CO release with a half-life of less than five min, is non-toxic, and suppresses LPS-induced nitrite generation model of inflammation in macrophages. The mechanism of action is very likely by the direct action of CO released from CORM-3 as was highlighted by the investigation into HO activity, HO-1 expression, iNOS expression and the use of SnPPIX. Further investigation may reveal greater promise for this first in line of the new CO-RMs, but, at a minimum highlights the extreme potential of the transition metal carbonyls as CO-RMs.

The study into the CO release of the new CO-RMs emphasizes the adaptive potential of the metal carbonyl complexes. Simple ligand addition and variation of metal centre results in an exciting portfolio of CO-RMs capable of a slow or fast kinetic of CO release, different solubilities in biological constituents and diverse biological activities.

It is this diversity and ability to refine CO-RMs that makes the development of a biologically compatible compound capable of therapeutic application an achievable goal. The development of CO-RMs has been requird for some time, considering the evolutionary importance of CO, and has opened up a window into understanding the biological function and possible therapeutic interventions of CO.

9 List of Publications

PUBLISHED ARTICLES

1) Sawle, P., Foresti, R., Green, C. J., & Motterlini, R. 2001, "Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia", *FEBS Lett.*, vol. 508, no. 3, pp. 403-406.

2) Motterlini, R., Sawle, P., Hammad, J., Bains, S., Alberto, R., Foresti, R., & Green, C. J. 2005, "CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule", *Faseb Journal*, vol. 19, no. 2, pp. 284-286.

3) Sawle, P., Foresti, R., Mann, B. E., Johnson, T. R., Green, C. J., & Motterlini, R. 2005, "Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages", *British Journal of Pharmacology*, vol. 145, no. 6, pp. 800-810.

4) Fairlamb I.J.S., Duhme-Klair A.K., Lynam J.M., Moulton B.E., O'Brien C.T., Whitwood A.C., Sawle P., Hammad J. and Motterlini R, 2006, "η^4-Pyrone metal carbonyl complexes as effective CO-releasing molecules (CO-RMs): importance of the 2-pyrone substituents", *Bioorg. Med. Chem. Lett.,* vol 16, no. 4, pp.995-998.

5) Sawle, P., Hammad, J., Fairlamb, I.J.S.,Moulton, B., O'Brien C.T., Lynam J.M., Duhme-Klair A.K., Foresti, R., Motterlini, R. 2006, "Bioactive properties of iron-containing carbon monoxide-releasing molecules (CO-RMs)", JPET. (In press).

Note:- The full articles can be found in the appendix (paragraph 11.5).

PUBLISHED ABSTRACTS

1) Motterlini R, Sawle P, Foresti R, Bassi R, and Green CJ. Homocysteine prevents NO-mediated induction of heme oxygenase-1 in vascular endothelial cells. In: ***Nitric Oxide***, 4(3):242, 2000.

2) Naughton, P., Foresti, R., Sawle, P., Green, C. J., & Motterlini, R. Induction of haem oxygenase-1 by nitroxyl anion (NO^-) in cardiomyocytes. J.Physiol. 531, P194. 2001.

CONGRESSES

1) Motterlini R, Sawle P, Foresti R, Bassi R, and Green CJ. Homocysteine prevents NO-mediated induction of heme oxygenase-1 in vascular endothelial cells. Congress: 1st International Conference on *Biology, Chemistry and Therapeutic Applications of Nitric Oxide*, San Francisco, California, June 3rd – 7th, 2000.

2) 1st International Conference on *Nitric Oxide Cell Survival and Cell Death*, Wolfson Institute for Biomedical Research, University College London, UK, September 14th – 15th, 2001.

3) 2nd International Conference on Heme Oxygenase (HO/CO) and Cellular Stress Response, Baia Verde Hotel, Catania, Italy, June 6th – 9th, 2002.

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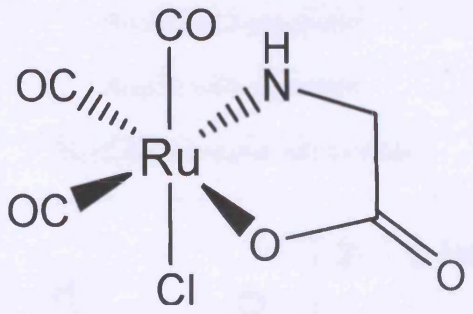
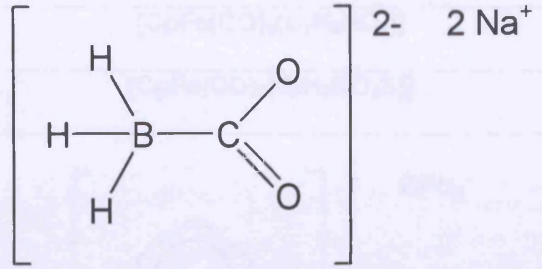
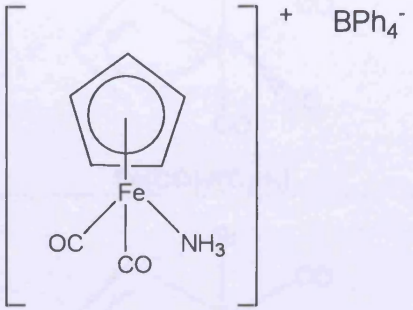
11 Appendices

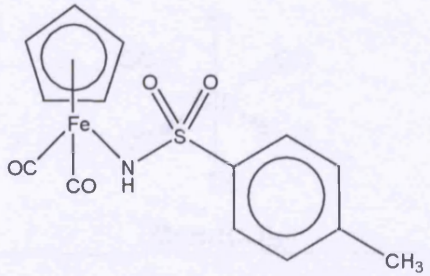
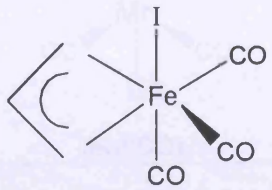
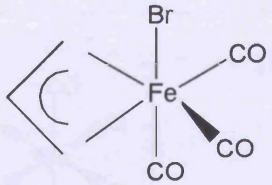
11.1 Appendix 1: Summary of CO-RMs Tested

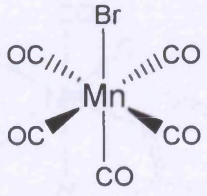
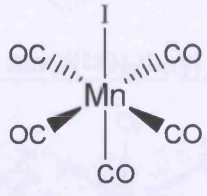
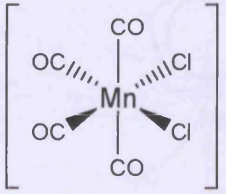
This section comprises a list of all the CO-RMs tested during my period of research. Structure, solubility and CO release are specified in all cases, while toxicity (determined by the Alamar blue assay) and vasorelaxtion (determined in an aortic rings model) are listed for selected CO-RMs. Although the screening of CO-RMs was routine it was necessary to identify suitable compounds with which to investigate biological activities. This is highlighted by the fact that from this large number of CO-RMs only a handful were chosen to test further.

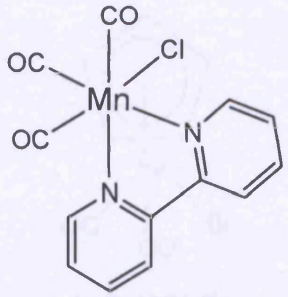
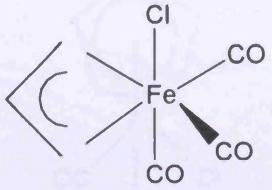
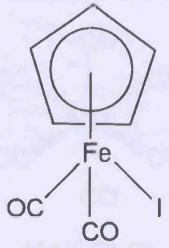
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-3F	 $Ru(CO)_3$-Cl-glycinato	294	Ru	Water	20	-	Instant	No	
CORM-3J	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	25	-	Instant	No	
CORM-3K	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	Old = 33 New =22	33 22	Instant	No	
CORM-3L	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	11	-	Instant	No	
CORM-3M	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	24	-	Instant	No	
CORM-3 3/12/03	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	30	30	Instant	No	
CORM-3 7/01/04	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	19	-	Instant	No	
CORM-3P	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	19	-	Instant	No	

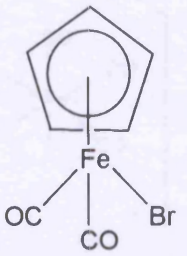
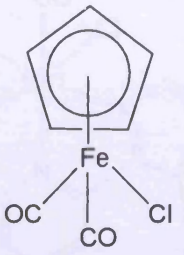
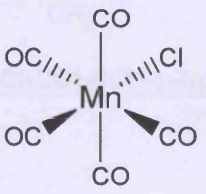
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-3T	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	24	-	Instant		
CORM-3N	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	46	53	Instant		
CORM-3U	$Ru(CO)_3$-Cl-glycinato	294	Ru	Water	37	50	Instant		
CORM-X1	Bis(2,4-dinitrophenyl) oxalate	422.2		DMSO	N/A	N/A	N/A	***	
CORM-A1	 $Na_2[H_3BCO_2]$	103.8	None	Water	8	34	Medium / Fast	No	+ +
CORM-A4			None	Water	0	0	N/A		
CORM-A5			None	Ethanol	0	0	N/A		
CORM-A6			None	Ethanol	0	0	N/A		
CORM-A7		108.12	None	Water	0	0	N/A		
CORM-A8		591	None	Water					

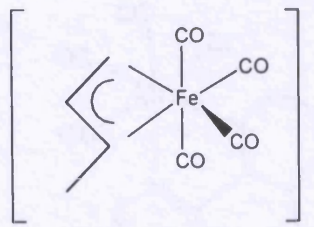
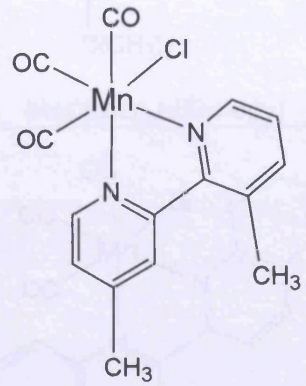
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-A9		95.8	None	Water	5	18 (60 mins)	Slow	No	
CORM-Z1	$[CpFe(CO)_2(C_8H_7N_2O_2)]$	338.1	Fe	Ethanol	0	0	N/A		+
CORM-Z2	$[CpFe(CO)_2(C_9H_8N_2O_2)]^+ [I]^-$	466	Fe	Water	>1	11	Slow / Medium	No	-
CORM-Z3	$[CpFe(CO)_2(C_4H_2NO_2)]$	273	Fe	Water	0	0	N/A	No	+ +
CORM-Z4	$[CpFe(CO)_2(C_4H_4NO_2)]$	275	Fe	Water	0	0	N/A		+
CORM-Z5	$[CpFe(CO)_2(C_6H_7NO_2S)]$	348.2	Fe	Water	>1	0	N/A		-
CORM-303	 $[CpFe(CO)_2(NH_3)]^+ BPh_4^-$	513	Fe	Ethanol	0	0	N/A	No	-

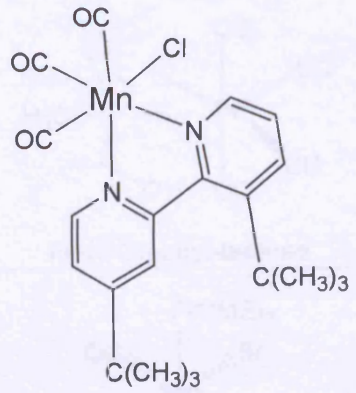
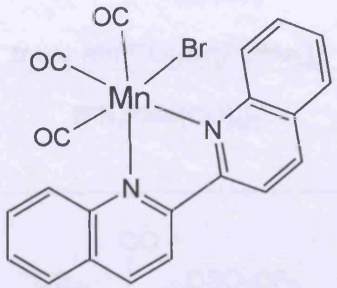
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-304	 [CpFe(CO)$_2$(para-tolyl-sulfonamide)]	347	Fe	Ethanol	>0.5	1	Slow	No	-
CORM-307	 $Fe(CO)_3I(C_3H_5)$	308	Fe	Ethanol	47	48	Instant	***	+ +
CORM-308	 $Fe(CO)_3Br(C_3H_5)$	261	Fe	Ethanol	47	48	Instant	***	+ +

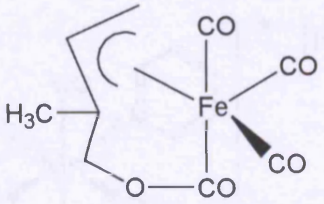
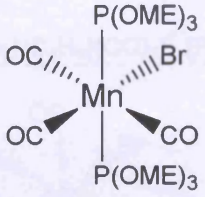
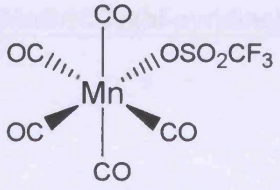
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-309	 $MnBr(CO)_5$	275	Mn	Ethanol	2	10	Slow	No	+
CORM-310	 $MnI(CO)_5$	322	Mn	Ethanol	0	8	Slow	No	-
CORM-311	$[Et_3NH][HFe_3(CO)11]$	496	Fe	Ethanol	10	28	Medium	No	+
CORM-312	 $[PPN]^+$ $(PPN)[Mn(CO)_4Cl_2]$	776.5	Mn	Ethanol	12	32	Slightly faster than 311	***	+

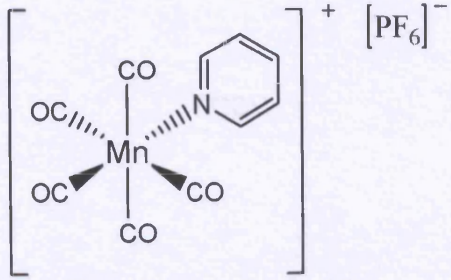
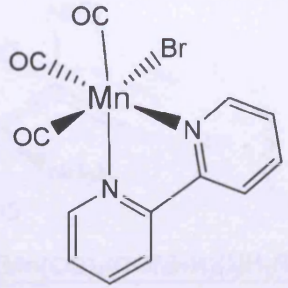
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-313	 $MnCl(CO)_3(bpy)$	330.6	Mn	Ethanol	>1	0	N/A	**	+ +
CORM-314	 $Fe(\eta\text{-}C_3H_5)(CO)_3Cl$	260.9	Fe	Ethanol	34	45	Medium / Fast	**	+ +
CORM-315	 $CpFe(CO)_2I$	303.9	Fe	Ethanol	0	11	Slow / Medium	No	-

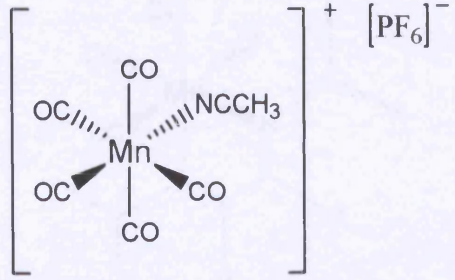
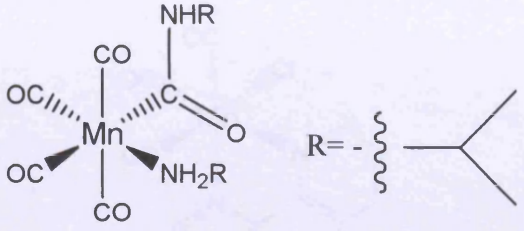
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-316	 $CpFe(CO)_2Br$	256.9	Fe	Ethanol	0	8	Slow / Medium	No	-
CORM-317	 $CpFe(CO)_2Cl$	212.4	Fe	Ethanol	0	6	Slow	No	-
CORM-318	 $Mn(CO)_5Cl$	230.4	Mn	Ethanol	>1	2	V Slow	No	+

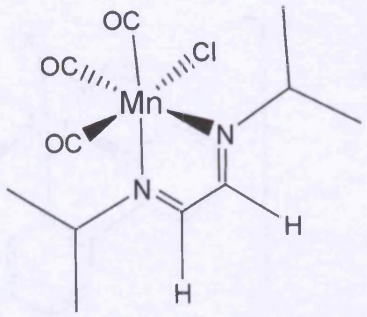
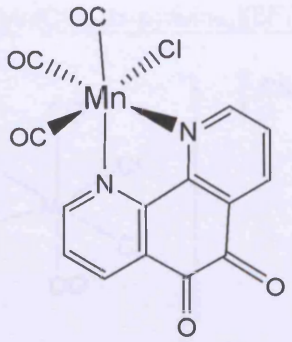
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-319	 $[BF_4]^-$ $[Fe(\eta\text{-}C_3H_5)(CO)_4][BF_4]$	352	Fe	Water	4	19	Slow / Medium	No	+
CORM-320	 $MnCl(CO)_3((CH_3)_2\text{-bpy})$	358.6	Mn	Ethanol	0	0	N/A	***	

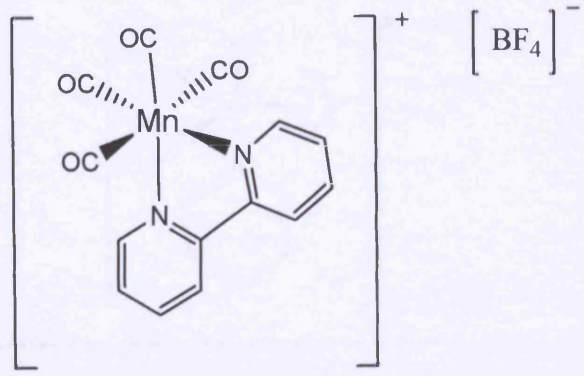
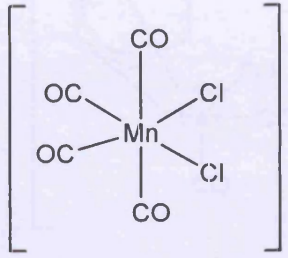
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-321	 $MnCl(CO)_3$(diBu-bpy)	442.6	Mn	Ethanol	0	0	N/A	***	
CORM-322	 $MnCl(CO)_3Br$(liquinoline)	420	Mn	Ethanol	0	0	N/A	*	

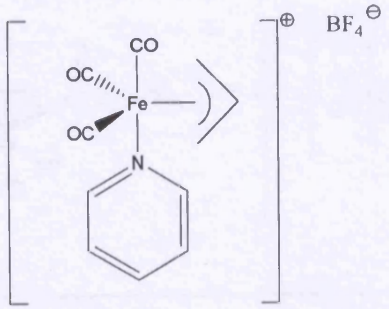
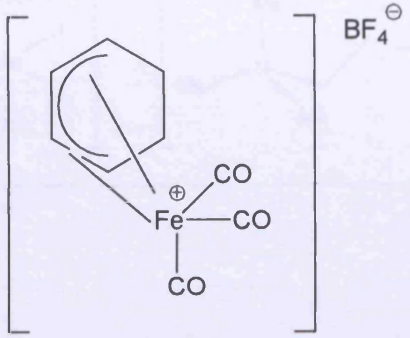
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-323	 Fe-$(CO)_3$-allyl-lactone	252	Fe	Ethanol	2	33	Slow / Medium	**	
CORM-324	 trans-$Mn(CO)_3Br[POMe_3]_2$	409	Mn	Ethanol	0	0	N/A	Slightly – not conc depen	
CORM-325	$[Ph_3PMn(CO)_4]_2$	864	Mn	Ethanol	0	0	N/A	No	
CORM-326	 $[Mn(CO)_5(OSO_2CF_3)]$	344	Mn	Ethanol (Sparingly in Water)	7	11 (60 mins)	Almost Instant	No	

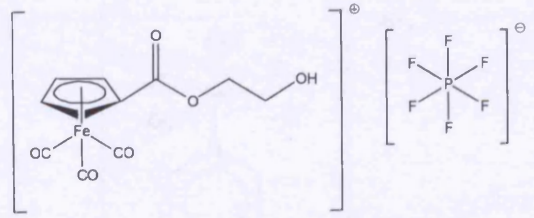
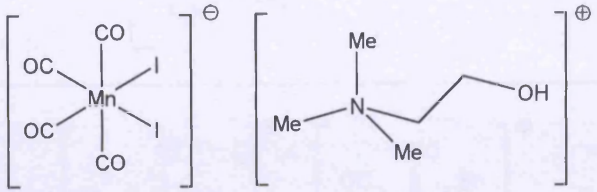
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-327	 $[PF_6]^-$ $[Mn(NC_5H_5)(CO)_5]^+ [PF_6]^-$	419	Mn	Ethanol	>1	>1	Almost Instant	No	
CORM-328	 $[MnBr(CO)_3(bi\text{-}pyridine)]$	375	Mn	Ethanol	>1	1.5	Almost Instant	**	

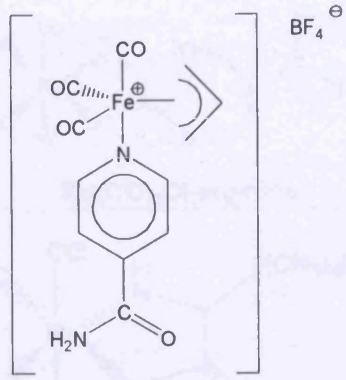
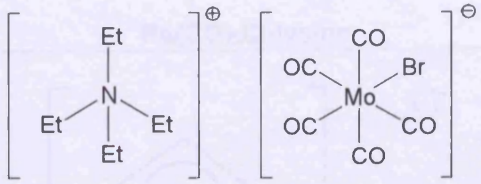
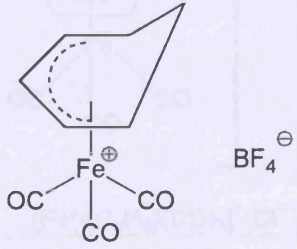
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-329	 $[Mn(CH_3CN)(CO)_5]^+ [PF_6]^-$	381	Mn	Ethanol	1.5	2.2	Almost Instant	No	
CORM-330	 $[Mn(CO)_4(CONHR)(NH_2R)]$	312	Mn	Ethanol	1.7	2.8	Almost Instant	No	

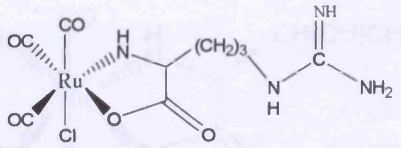
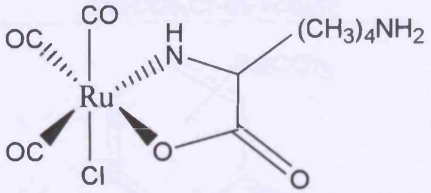
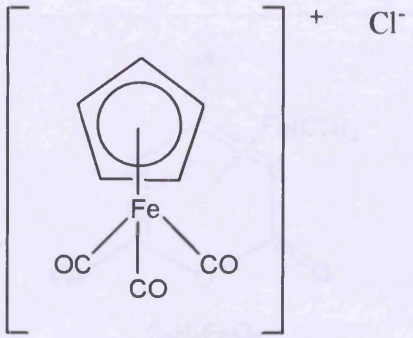
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-331	 $Mn(CO)_3Cl(^iPr\text{-}DAB)$	314.65	Mn	Ethanol	0	0	N/A		
CORM-332	 $MnCl(CO)_3(dpq)$	384.61	Mn	Ethanol	0	0	N/A		

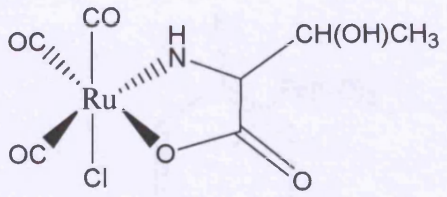
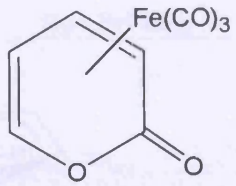
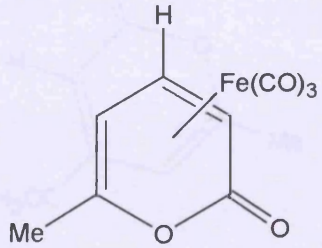
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-333	 $[BF_4]^-$ $[Mn(CO)_4(bi\text{-}pyridine)][BF_4]$	409.97	Mn	Water	0	>1	N/A		
CORM-334	 [Choline] $[Choline][Mn(CO)_4Cl_2]$	341	Mn	Water	31	40	Fast	No	

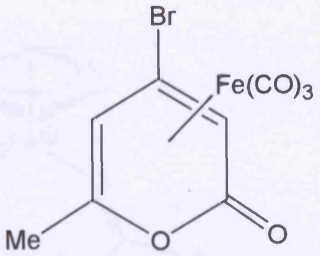
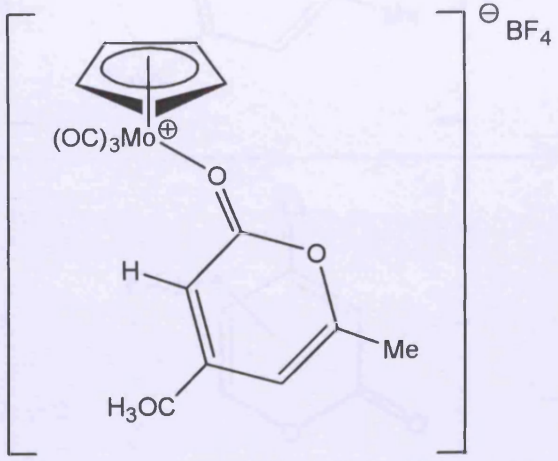
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-335		347	Fe	Water	10	19	Slow	*	
CORM-336	 $[Fe(CO)_3(C_7H_9)]BF_4$	320	Fe	Water	0	1	Slow		

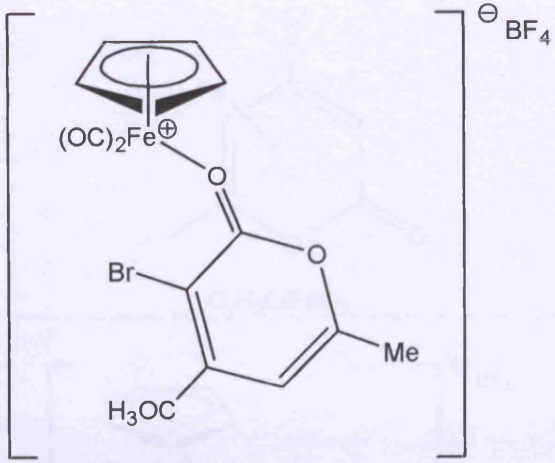
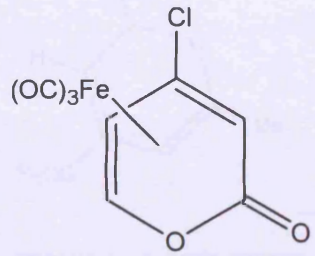
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-337		438	Fe	Water	12	22	Slow	No	
CORM-338		525	Mn	Water	4	14	Slow	No	

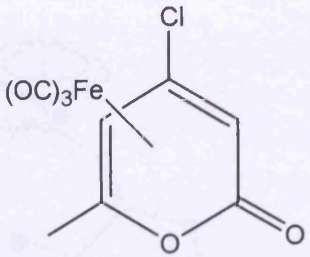
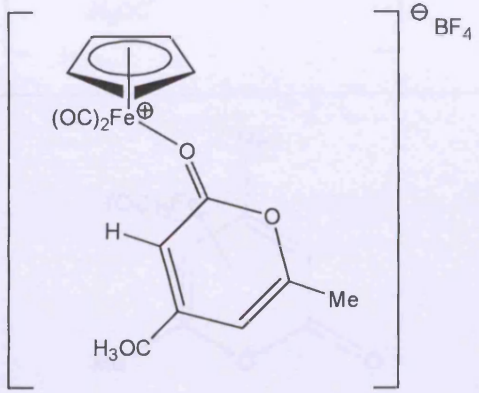
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (μM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-339		390	Fe	Water	7	12	Slow	No	
CORM-340		446	Mo	Water	8	37	Medium	**	
CORM-341		306	Fe	Water	0	0	N/A		

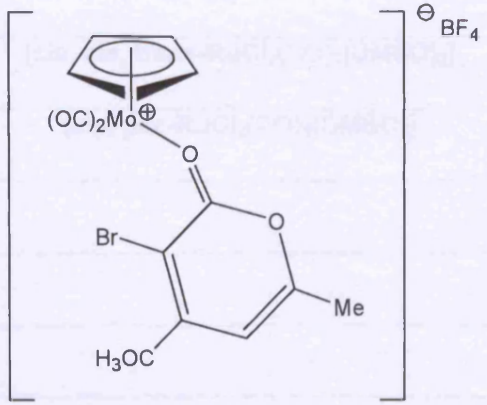
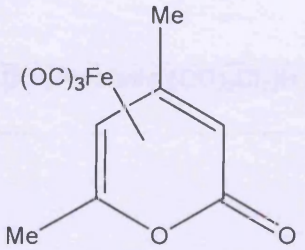
Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-43	 $Ru(CO)_3Cl$-arginine	392	Ru	Water	26	27	Almost Instant	No	
CORM-46	 $Ru(CO)_3Cl$-lysine	364	Ru	Water	42	42	Instant	No	
CORM-70	 $[Fe(\eta C_5H_5)(CO)_3]^+ Cl^-$	240.5	Fe	DMSO	2	17	Medium / Fast	No	+ +

Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-74	 $Ru(CO)_3Cl$-threonine	337	Ru	Water	36	36	Instant	No	
CORM-F1		236	Fe	Water	0	0	N/A		
CORM-F2	 $C_9H_6FeO_5$	250	Fe	DMSO	0	0	N/A		-

Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-F3	 $C_9H_5BrFeO_5$	329	Fe	DMSO	2	15	Medium / Fast	200 µM	+ +
CORM-F4			Mo		7	12	Slow		
CORM-F5									

Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-F6			Fe		0.5	1	Slow		
CORM-F7	 $C_8H_3ClFeO_5$	270.4	Fe	DMSO	0	0.5	Slow	200 µM	

Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-F8	 $C_9H_5ClFeO_5$	284.43	Fe	DMSO	1	4.5	Slow	200 µM	
CORM-F9		403	Fe	DMSO	0.7	4	Slow	200 µM	

Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-F10		551	Mo	DMSO	8	50	Fast	200 µM	
CORM-F11		262	Fe	DMSO	0	0	N/A	200 µM	
CORM-1CO	[Na] [t-$RuCl_4CO(DMSO)$]	372.011	Ru	Water	0	0	N/A		
CORM-2CO	cis, cis, trans-$RuCl_2(DMSO)_2(DMSO)CO$	434.37	Ru	Water	>1	>1	N/A		

Code	Formula / Structure	Molecular Weight (Theor)	Transition Metal	Solubility	CO Release (µM)		Kinetics of CO Release[1]	Toxic effect on cells[3]	Vessel Relaxation[2]
					5 min	90 min			
CORM-3CO	[cis, cis, cis $RuCl_2(CO)_2(DMSO)(DMSO)$]	384.25	Ru	Water	8	9	Instant		
CORM-4CO	[cis, cis, trans-$RuCl_2(CO)_2(DMSO)_2$]	384.25	Ru	Water	17.5	19	Instant		
CORM-5CO	[cis, pac-$RuCl_2(CO)_3(DMSO)$]	334.13	Ru	Water	20	31	Medium		
CORM-Z1		338.1		Ethanol	0	0	Slow		
CORM-Z2		466		Water	0	11	Slow		
CORM-Z3		273		Water	0	0	Slow		
CORM-Z4		275		Water	0	0	Slow		
CORM-Z5		348.2		Water	0	0	Slow		
CORM-Picolinic	[$Ru(Picolinic)(CO)_2Cl_2$]H	351.17	Ru	Water	3	3	Slow		

Key:

(1) The myoglobin (Mb) assay, which quantifies the production of MbCO from deoxy-Mb, was used to measure the rate of CO released over time in order to classify the compounds as fast (F) or slow (S) releasers of CO. Forty micromolar (40 μM) of each compound were added to a solution containing 40 μM Mb.

(2) The experiments using the isolated aortic rings were conducted to assess the extent of vasorelaxation. One hundred micromolar (100 μM) of each compound were added to a pre-contracted ring and vasorelaxation was assessed as a percentage of the initial contraction, which was expressed as good (+) or very good (++). The sign – indicates that no relaxation was detected.

(3) Cytotoxicity was measured in RAW264.7 macrophages incubated for 24 h with 10, 50 or 100 μM of each compound. The loss in cell viability was measured as a percentage of control. * indicates toxicity detected at 100 μM; ** indicates toxicity detected at 50 μM; *** indicates toxicity detected at 10 μM; V indicates that cells were viable and no toxicity was detected up to 100 μM.

11.2 Appendix 2: The Effect of CORM-2 and CORM-3 on Human Blood Aggregation

11.2.1 Introduction

It has been shown in a number of studies that CO has an important role to play in the circulatory system. The effects of CO on blood pressure may be regulated directly through the promotion of vasodilation or indirectly via acting on the nucleus tractus solitaius which alters glutamatergic transmission and ultimately lowers blood pressure (Maines 1997;Johnson, Kozma, & Colombari 1999;Morse & Sethi 2002). The ablumenal release of CO by endothelial cells may play an important fundamental role in regulating blood flow by inhibiting smooth muscle tone and SMC proliferation (Morita, Perrella, Lee, & Kourembanas 1995). It has also been found that CO released into the vessel lumen may modulate blood fluidity by preventing the aggregation and adhesion of platelets (Morita, Perrella, Lee, & Kourembanas 1995). With the long term goal of CO-RM reasearch and development being the creation of a pharmacologically viable agent this last action of CO posed an interesting question as to the effect CO-RMs may have on blood. Would CO-RMs have the potential to prevent platelet aggregation in human blood?

11.2.2 Materials and Methods

Whole blood (50 ml) was aquired from healthy adult human volunteers and added to a tube containing EDTA to prevent coagulation. Blood was then either used to prepare platelet rich plasma (PRP) or used directly as whole blood. PRP was prepared by centrifuging whole blood for 10 min at 100 x g after which the yellow top layer (PRP) was removed being careful not to disturb the red blood cell layer underneath. The number of platelets in the PRP was then

counted using a haemocytometer and the dilution factor required to give an overall platelet concentration of 10^8 platelets per ml determined. To adjust the platelet levels the PRP was spun for 5 min at 1000 x g to obtain a platelet pellet. This pellet was then resuspended using enough saline (0.9 % w/v) to attain 10^8 platelets per ml.

Blood aggregation was determined using a dual channel Chrono-log Whole Blood Aggregometer (USA) as follows. Either 1 ml PRP or 500 µl whole blood + 500 µl saline was added to a cuvette containg an iron bar stirrer. This was then placed into the device where it was warmed and stirred for 10 min. The probe was then added, which measures aggregation by the impedence to electrical current passed between two metal wires. Having allowed a brief period for equilibration, the sample was set to baseline by holding the baseline button and setting the chart recorder to zero, the sample was now ready to test. Initially the extent to which the stock whole blood or PRP aggregates was tested by adding 10 µM of adenosine diphosphate (from a 10 mM stock) to the sample and leaving it for 5 minutes. This gives us the maximal aggregation that can be achieved by the current PRP or whole blood stock. The process was then repeated adding CO-RMs prior to adensosine diphosphate treatment to determine if any the effect of CO-RMs on aggregation.

11.2.3 Results

The following section briefly presents all the experiments carried out using the whole blood aggregometer. Aggregation is determined as the difference in ohmes between blood or PRP treated with adenosine diphosphate alone and blood or PRP pretreated with CO-RMs.

Platelet Studies - Using the Whole Blood Aggregometer (WBA)

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
Chart Recorder Data						
07/09/2001	WB	AD	10	Blood treated solely with AD	35	n/a
07/09/2001	WB	CORM-2	50	1 min preinc with CORM then 10μM AD added	29	82.9
07/09/2001	WB	CORM-2	20	1 min preinc with CORM then 10μM AD added	35	100
10/09/2001	WB	AD	10	Blood treated solely with AD	20	n/a
10/09/2001	WB	AD	10	Blood treated solely with AD	26	n/a
10/09/2001	WB	AD	10	Blood treated solely with AD	31	n/a
10/09/2001	WB	AD	10	Blood treated solely with AD	27	n/a
10/09/2001	WB	CORM-3	300	1 min preinc with CORM-3and then 10μM AD added	30	115.4
10/09/2001	WB	CORM-3	200	1 min preinc with CORM-3and then 10μM AD added	19	73.1
10/09/2001	WB	CORM-3	100	1 min preinc with CORM-3and then 10μM AD added	40	153.8
10/09/2001	WB	CORM-3	300	1 min preinc with CORM-3and then 10μM AD added	20	76.9
11/09/2001	WB	CORM-3	300	10 min preinc with CORM-3and then 10μM AD added	16	47.1
11/09/2001	WB	CORM-3	200	10 min preinc with CORM-3and then 10μM AD added	17	50
11/09/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	20	58.8
11/09/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	17	50
11/09/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	14	41.2
11/09/2001	WB	AD	10	Blood treated solely with AD	30	n/a
11/09/2001	WB	AD	10	Blood treated solely with AD	34	n/a
11/09/2001	WB	AD	10	Blood treated solely with AD	36	n/a
11/09/2001	WB	AD	10	Blood treated solely with AD	36	n/a
29/10/2001	WB	AD	10	Blood treated solely with AD	35	n/a

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
29/10/2001	WB	Thrombin	1U/ml	Blood treated solely with Thrombin	38	n/a
02/11/2001	WB	AD	10	Blood treated solely with AD	25	n/a
02/11/2001	WB	CORM-3	200	10 min preinc with CORM-3and then 10μM AD added	13	65
02/11/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	21	105
02/11/2001	WB	AD	10	Blood treated solely with AD	16	n/a
02/11/2001	WB	AD	10	Blood treated solely with AD	20	n/a
02/11/2001	WB	AD	10	Blood treated solely with AD	25	n/a
02/11/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	11	48.7
02/11/2001	WB	AD	10	Blood treated solely with AD	27	n/a
02/11/2001	WB	CORM-3	25	10 min preinc with CORM-3and then 10μM AD added	error	
02/11/2001	WB	AD	10	Blood treated solely with AD	16	n/a
02/11/2001	WB	AD	10	Blood treated solely with AD	30	n/a
02/11/2001	WB	CORM-3	25	10 min preinc with CORM-3and then 10μM AD added	20	61.5
02/11/2001	PRP	CORM-3	25	10 min preinc with CORM-3and then 10μM AD added	36	85.7
02/11/2001	WB	AD	10	Blood treated solely with AD	35	n/a
02/11/2001	WB	AD	10	Blood treated solely with AD	29	n/a
02/11/2001	WB	CORM-3	75	10 min preinc with CORM-3and then 10μM AD added	21	72.4
02/11/2001	PRP	AD	10	Blood treated solely with AD	42	n/a
02/11/2001	PRP	CORM-3	75	1 min preinc with CORM-3and then 10μM AD added	37	88.1
02/11/2001	WB	CORM-3	25	3 min preinc with CORM-3and then 10μM AD added	38	131
08/11/2001	WB	AD	10	Blood treated solely with AD	25	n/a
08/11/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	14	65.1
08/11/2001	WB	SN	50	10 min preinc with SN and then 10μM AD added	18	83.7
08/11/2001	WB	AD	10	Blood treated solely with AD	15	n/a

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
08/11/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	15	61.7
08/11/2001	WB	AD	10	Blood treated solely with AD	20	n/a
08/11/2001	WB	SN	100	10 min preinc with SN and then 10μM AD added	14	57.6
08/11/2001	WB	AD	10	Blood treated solely with AD	28	n/a
08/11/2001	WB	AD	10	Blood treated solely with AD	21	n/a
08/11/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	23	109.5
08/11/2001	WB	SN	50	10 min preinc with SN and then 10μM AD added	13	61.9
08/11/2001	WB	SN	10	10 min preinc with SN and then 10μM AD added	15	71.4
08/11/2001	WB	AD	10	Blood treated solely with AD	21	n/a
08/11/2001	WB	iCORM-3	50	10 min preinc with iCORM-3 and then 10μM AD added	24	77.4
08/11/2001	WB	AD	10	Blood treated solely with AD	32	n/a
08/11/2001	WB	CORM-2	50	10 min preinc with CORM-2 and then 10μM AD added	18	58.1
08/11/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	20	64.5
08/11/2001	WB	AD	10	Blood treated solely with AD	30	n/a
Computer Data						
14/11/2001	WB	AD	10	Blood treated solely with AD	15	n/a
14/11/2001	WB	iCORM-3	10	10 min preinc with iCORM-3 and then 10μM AD added	15	100
14/11/2001	WB	iCORM-3	50	10 min preinc with iCORM-3 and then 10μM AD added	18	120
14/11/2001	WB	iCORM-3	100	10 min preinc with iCORM-3 and then 10μM AD added	18	120
14/11/2001	WB	iCORM-3	100	10 min preinc with iCORM-3 and then 10μM AD added	16	106.7
14/11/2001	WB	iCORM-3	150	10 min preinc with iCORM-3 and then 10μM AD added	5	33.3
14/11/2001	WB	AD	10	Blood treated solely with AD	14	n/a
14/11/2001	WB	CORM-3	10	10 min preinc with CORM-3and then 10μM AD added	18	128.6
14/11/2001	WB	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	20	142.9
14/11/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	20	142.9
14/11/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	18	128.6

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
27/11/2001	WB	AD	10	Blood treated solely with AD	10	n/a
27/11/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	10	100
27/11/2001	WB	AD	10	Blood treated solely with AD	5	n/a
27/11/2001	WB	AD	10	Blood treated solely with AD	10	n/a
27/11/2001	WB	AD	10	Blood treated solely with AD	10	n/a
27/11/2001	WB	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	10	100
27/11/2001	WB	AD	10	Blood treated solely with AD	10	n/a
04/12/2001	PRP	AD	10	Blood treated solely with AD	15	n/a
04/12/2001	PRP	iCORM-3	100	10 min preinc with iCORM-3 and then 10μM AD added	15	100
04/12/2001	PRP	SN	100	10 min preinc with SN and then 10μM AD added	0	0
04/12/2001	PRP	AD	10	Blood treated solely with AD	16	n/a
04/12/2001	PRP	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	5	31.3
04/12/2001	PRP	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	18	112.5
04/12/2001	PRP	CORM-3	50	10 min preinc with CORM-3and then 10μM AD added	19	172.7
04/12/2001	PRP	CORM-3	100	10 min preinc with CORM-3and then 10μM AD added	19	172.7
04/12/2001	PRP	AD	10	Blood treated solely with AD	11	n/a
04/12/2001	PRP	SN	50	10 min preinc with SN and then 10μM AD added	0	0
04/12/2001	PRP	iCORM-3	100	10 min preinc with iCORM-3 and then 10μM AD added	5	31.3
13/12/2001	WB	AD	10	Blood treated solely with AD	20	n/a
13/12/2001	WB	TJ35	100	10 min preinc with TJ35 and then 10μM AD added	17	85
13/12/2001	WB	TJ35	200	10 min preinc with TJ35 and then 10μM AD added	17	85

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
13/12/2001	WB	SN	20	10 min preinc with SN and then 10μM AD added	0	0
13/12/2001	WB	SN	10	10 min preinc with SN and then 10μM AD added	0	0
18/12/2001	PRP	AD	10	Blood treated solely with AD	20	n/a
18/12/2001	PRP	TJ35	100	10 min preinc with TJ35 and then 10μM AD added	20	100
18/12/2001	PRP	TJ35	100	5 min preinc with TJ35 and then 10μM AD added	19	95
18/12/2001	PRP	TJ35	200	5 min preinc with TJ35 and then 10μM AD added	20	100
18/12/2001	PRP	AD	10	Blood treated solely with AD	16	n/a
18/12/2001	PRP	TJ36	100	10 min preinc with TJ36 and then 10μM AD added	17	130.8
18/12/2001	PRP	TJ36	100	5 min preinc with TJ36 and then 10μM AD added	18	138.5
18/12/2001	PRP	TJ36	200	5 min preinc with TJ36 and then 10μM AD added	16	123.1
18/12/2001	PRP	AD	10	Blood treated solely with AD	10	n/a
21/01/2002	PRP	AD	10	Blood treated solely with AD	19	n/a
21/01/2002	PRP	TJ35	100	10 min preinc with TJ35 and then 10μM AD added	19	100
21/01/2002	PRP	TJ35	100	10 min preinc with TJ35 and then 10μM AD added	17	89.5
21/01/2002	PRP	AD	10	Blood treated solely with AD	17	n/a
21/01/2002	PRP	TJ35	200	10 min preinc with TJ35 and then 10μM AD added	18	105.9
21/01/2002	PRP	TJ35	200	10 min preinc with TJ35 and then 10μM AD added	17	100
21/01/2002	PRP	AD	10	Blood treated solely with AD	23	n/a
21/01/2002	PRP	TJ36	100	10 min preinc with TJ36 and then 10μM AD added	22	95.7
21/01/2002	PRP	TJ36	100	10 min preinc with TJ36 and then 10μM AD added	21	91.3
21/01/2002	PRP	AD	10	Blood treated solely with AD	24	n/a
21/01/2002	PRP	TJ36	200	10 min preinc with TJ36 and then 10μM AD added	22	91.7
21/01/2002	PRP	TJ36	200	10 min preinc with TJ36 and then 10μM AD added	20	83.3
29/01/2002	PRP	AD	10	Blood treated solely with AD	18	n/a
29/01/2002	PRP	TJ36	100	2 min preinc with TJ36 and then 10μM AD added	18	100.0

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
29/01/2002	PRP	TJ36	100	2 min preinc with TJ36 and then 10μM AD added	18	100.0
29/01/2002	PRP	AD	10	Blood treated solely with AD	23	n/a
29/01/2002	PRP	SNP	100	2 min preinc with SNP and then 10μM AD added	4	17.4
29/01/2002	PRP	SNP	100	2 min preinc with SNP and then 10μM AD added	0	0.0
29/01/2002	PRP	AD	2	Blood treated solely with AD	21	n/a
08/02/2002	PRP	AD	0.1	Blood treated solely with AD	0	n/a
08/02/2002	PRP	AD	1	Blood treated solely with AD	20	n/a
08/02/2002	PRP	AD	0.5	Blood treated solely with AD	5	n/a
08/02/2002	PRP	AD	0.5	Blood treated solely with AD	0	n/a
08/02/2002	PRP	TJ35	100	10 min preinc with TJ35 and then 1μM AD added	17	85
08/02/2002	PRP	AD	1	Blood treated solely with AD	20	n/a
08/02/2002	PRP	AD	0.1	Blood treated solely with AD	0	n/a
08/02/2002	PRP	AD	1	Blood treated solely with AD	20	n/a
08/02/2002	PRP	AD	0.5	Blood treated solely with AD	0	n/a
08/02/2002	PRP	AD	0.5	Blood treated solely with AD	5	n/a
08/02/2002	PRP	TJ36	100	10 min preinc with TJ36 and then 1μM AD added	15	69.8
08/02/2002	PRP	AD	1	Blood treated solely with AD	23	n/a
12/02/2002	PRP	AD	1	Blood treated solely with AD	19	n/a
12/02/2002	PRP	TJ35	100	10 min preinc with TJ35 and then 1μM AD added	19	100
12/02/2002	PRP	TJ35	100	10 min preinc with TJ35 and then 1μM AD added	18	94.7
12/02/2002	PRP	AD	1	Blood treated solely with AD	17	n/a
12/02/2002	PRP	AD	1	Blood treated solely with AD	17	n/a
12/02/2002	PRP	TJ35	200	10 min preinc with TJ35 and then 1μM AD added	17	17
12/02/2002	PRP	TJ35	200	10 min preinc with TJ35 and then 1μM AD added	16	94.1
12/02/2002	PRP	TJ35	100	TJ35 added and then 1μM AD added immediately afterwards	18	?

Date	Sample Type	Compound	Conc (μM)	Treatment	Max Ohms	aggreagation ~%
12/02/2002	PRP	AD	1	Blood treated solely with AD	22	n/a
12/02/2002	PRP	TJ36	100	10 min preinc with TJ36 and then 1μM AD added	20	90.9
12/02/2002	PRP	TJ36	100	10 min preinc with TJ36 and then 1μM AD added	19	86.4
12/02/2002	PRP	AD	1	Blood treated solely with AD	20	n/a
12/02/2002	PRP	TJ36	200	10 min preinc with TJ36 and then 1μM AD added	20	100.0
12/02/2002	PRP	TJ36	200	10 min preinc with TJ36 and then 1μM AD added	S.A.	n/a
12/02/2002	PRP	TJ36	200	10 min preinc with TJ36 and then 1μM AD added	20	100.0
19/02/2002	PRP	AD	1	Blood treated solely with AD	19	n/a
19/02/2002	PRP	TJ35	100	5 min preinc with TJ35 and then 1μM AD added	10	52.6
19/02/2002	PRP	TJ35	100	5 min preinc with TJ35 and then 1μM AD added	19	100.0
19/02/2002	PRP	AD	1	Blood treated solely with AD	19	n/a
19/02/2002	PRP	TJ35	200	5 min preinc with TJ35 and then 1μM AD added	12	68.6
19/02/2002	PRP	TJ35	200	5 min preinc with TJ35 and then 1μM AD added	15	85.7
19/02/2002	PRP	AD	1	Blood treated solely with AD	16	n/a
19/02/2002	PRP	AD	1	Blood treated solely with AD	15	n/a
19/02/2002	PRP	TJ36	100	5 min preinc with TJ36 and then 1μM AD added	18	102.9
19/02/2002	PRP	TJ36	100	5 min preinc with TJ36 and then 1μM AD added	20	114.3
19/02/2002	PRP	AD	1	Blood treated solely with AD	20	n/a
19/02/2002	PRP	TJ36	200	5 min preinc with TJ36 and then 1μM AD added	6	32.4
19/02/2002	PRP	TJ36	200	5 min preinc with TJ36 and then 1μM AD added	17	91.9
19/02/2002	PRP	AD	1	Blood treated solely with AD	17	n/a
27/02/2002	PRP+T	AD	1	Blood treated solely with AD	0	n/a
27/02/2002	PRP+T	AD	10	Blood treated solely with AD	0	n/a
27/02/2002	PRP+T	AD	1	Blood treated solely with AD	0	n/a
27/02/2002	PRP+T	AD	10	Blood treated solely with AD	0	n/a

Date	Sample Type	Compound	Conc (µM)	Treatment	Max Ohms	aggreagation ~%
26/03/2002	PRP+T	AD	1	Blood treated solely with AD	0	n/a
26/03/2002	PRP+T	AD	10	Blood treated solely with AD	14	n/a
26/03/2002	PRP+T	AD	5	Blood treated solely with AD	7	n/a
26/03/2002	PRP+T	AD	10	Blood treated solely with AD	21	n/a
26/03/2002	PRP+T	AD	1	Blood treated solely with AD	4	n/a

Key:
WB = Whole Blood
PRP = Platelett Rich Plasma
T = Tyrodes Buffer
AD = Adenosine Diphosphate
SN = Sodium Nitroprusside
S.A. = Spontaneous Aggregation

11.2.4 Discussion

The data obtained in this particular study using CO-RMs was inherently inconsitent. Overall there was a trend that suggested CO-RMs did attenuate aggregation; however, this was neither significant nor consistent. Experiments carried out using the NO donor sodium nitroprusside (SNP) proved to be far more reliable and demonstrated a significant, consistent and reproducable inhibition of ADP associated aggregation. The preparation of PRP caused further problems since after collecting blood there is only a 3 hour period in which the aggregating ability is retained. To generate PRP took nearly 2 hours and often yielded very small volumes compared to the original amount of blood collected. This data neither supports nor disputes the ability of CO to inhibit blood aggregation. It also does not answer the question of whether CO-RMs can replicate the action of CO in blood since any effects observed were difficult to reproduce. With the potency of NO donor SNP to inhibit aggregation it may be the CO-RMs are just unable to elicit a response. However, it may also be possible that the assay needs to be refined possibly by warming CO-RMs prior to addition since in these early CO-RM experiments they were kept on ice. Needless to say there is still much to investigate and elucidate from further studies on CO-RMs and their effect on blood aggregation.

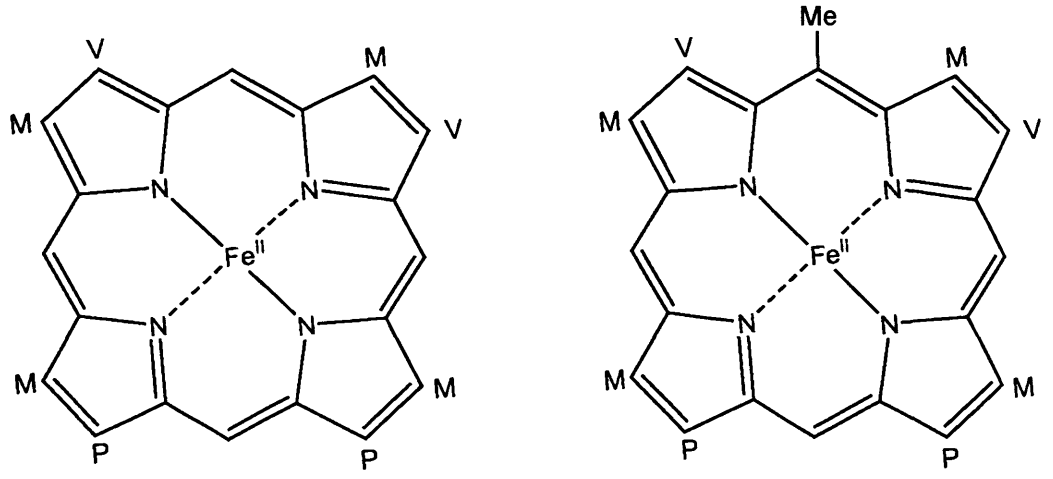
11.3 Appendix 3: Preliminary Tests Using Alpha-*Meso*-Methyl-Haem a Novel Haem Oxygenase Substrate that does not Generate CO

11.3.1 Introduction

Haem oxygenase (HO) is the rate limiting step in the catabolism of haem. The products of its activity are Fe(III), carbon monoxide (CO) and biliverdin which goes on to be catabolised further to bilirubin by biliverdin reductase. Biliverdin, bilirubin and CO have all in the last decade or so been shown to be involved in the cytoprotective effects associated with an increase in HO activity and expression. Both biliverdin and bilirubin are powerful antioxidants while CO has been shown to be anti-inflammatory, anti-apoptotic, vasodilatory and involved in cytoprotection in ischaemia/repurfusion injury to name but a few of its functions. The contribution of each of these products of HO activity has been investigated by the addition of each compound individually and in comibantion with one another. Obviously this is not an ideal way to investigate the various effects each has since the use of these compounds in combination does not accurately reflect what happens physiologically in terms of the final concentrations of each. The use of a haem isomer, α-*meso*-methyl-haem (αMMH), which when catabolised by HO does not go on to generate CO lends itself well to studying the end products of HO activity in a more biologically relevant manner (Figure 11.1). Normally the oxidation of haem by HO results in regiospecific elimination of the α-*meso*-carbon as CO and the formation of biliverdin IXα. It is generally accepted that this reaction proceeds via the α-*meso*-hydoxylation of haem. Torpey & Ortiz de Montellano found that HO-1 can oxidise an α-*meso*-methyl-substituted haem to biliverdin IXα without proceeding via the α-*meso*-hydroxy

intermediate and as such does not generate CO (Torpey & Ortiz de Montellano 1996).

This novel compound also enables a unique opputunity to use a newly synthesised group of compounds developed by our group to replace the lost CO function. Carbon monoxide-releasing molecules (CO-RMs) are a group of compounds able to release CO in biological buffers to simulate endogenous CO release. Depending on the structure of the CO-RM the kinetics and the amount of CO released can be altered to suit the desired action. In this instance the comparison of cells treated with αMMH or hemin can allow us to investigate the contribution of biliverdin/bilirubin in the presence or absence of CO, whilst, CO-RMs can enable us to replace the lost function of CO.



Haem

α-*meso*-methyl-haem

Figure 11.1:- Structural differences between haem and α-*meso*-methyl-haem (αMMH).
The presence of a methyl substituent in αMMH prevents α-*meso*-hydroxylation of haem as demonstrated by the failure of αMMH to generate CO when catabolised by HO-1.

11.3.2 Material and Methods

αMMH was prepared as per the protocol outlined by Torpey & Ortiz de Montellano (Torpey & Ortiz de Montellano 1996). It was prepared fresh prior to each experiment as a final stock concentration of 1.5 mM in 1 ml of dH_2O and 5 µl of 2 M NaOH. Bovine aortic endothelial cells (BAEC) were treated for 6 h in the presence of 5, 25 and 50 µM αMMH or hemin to investigate the effect on HO activity.

HO activity was determined as follows. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH = 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 µM). The reaction was conducted at 37 °C in the dark for 1 h and terminated by the addition of 1 ml chloroform; the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

11.3.3 Results

Both αMMH and hemin caused a concentration dependent increase in the level of HO activity. Interestingly, the use of hemin (at all concentrations) significantly increased HO activity compared to an equilivant αMMH concentration. This was most evident at 5 µM where αMMH did not increase HO activity significantly compared to control but hemin did.

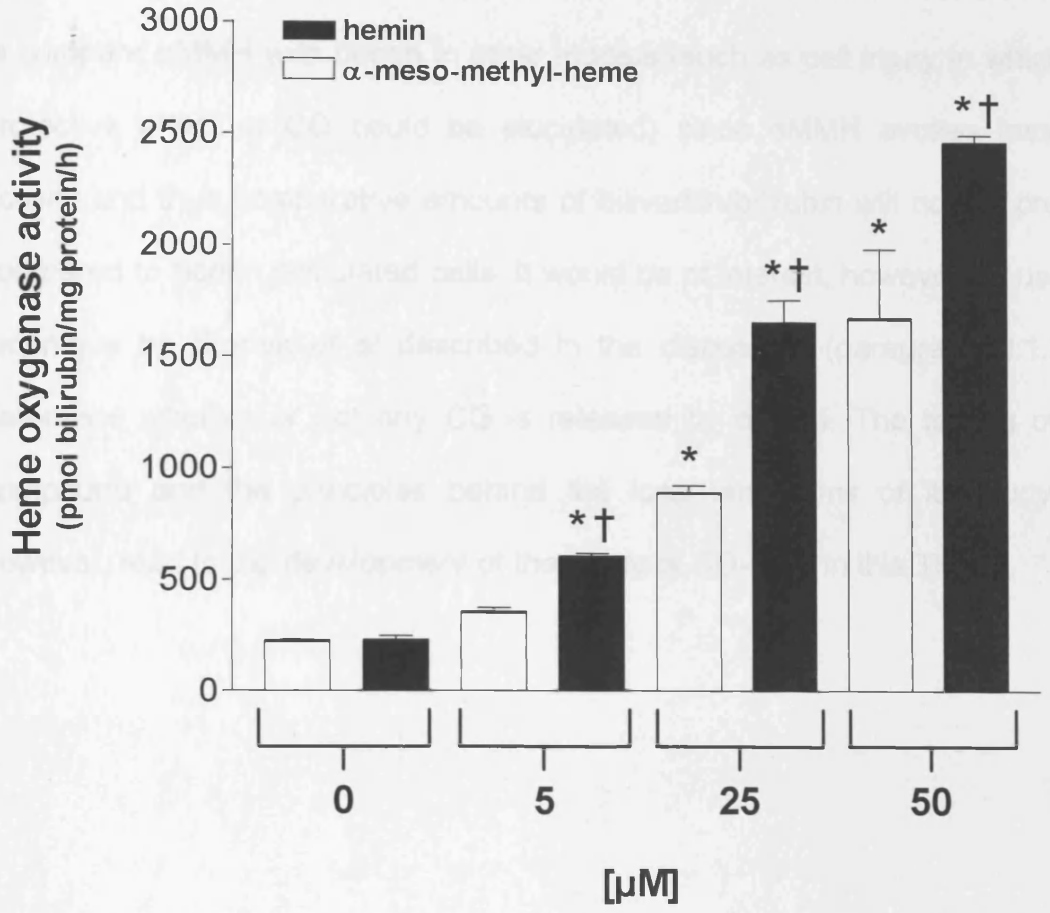


Figure 11.2:- Effect of hemin and αMMH on HO activity in bovine aortic endothelial cells.
Bovine aortic endothelial cells (BAEC) were incubated with increasing concentrations of αMMH (5-50 μM) or hemin (5-50 μM). Haem oxygenase activity was determined 6 h after exposure to the different concentrations. Data represent the mean ± S.E.M. of 6 independent experiments. * indicates $P < 0.05$ vs. control (0); † indicates $P < 0.05$ vs. αMMH.

11.3.4 Discussion

The treatment of BAEC with αMMH resulted in a less of an increase in HO activity compared to cells treated with hemin. The way in which HO utilises αMMH may be responsible for the reduction in activity since it may be less likely to induce HO protein expression. As such, it would be of interest to compare the effect of hemin and αMMH on HO-1 protein expression via Western blot. It is also likely that the generation of CO, which can go on to further stimulate HO

induction, is responsible. The reduction in activity with αMMH makes it difficult to compare αMMH with hemin in other models (such as cell injury in which the protective effect of CO could be elucidated) since αMMH evokes less HO activity and thus comparative amounts of biliverdin/bilirubin will not be present compared to hemin stimulated cells. It would be of interest, however, to use the technique by Stonek *et al* described in the discussion (paragraph 8.1.2) to determine whether or not any CO is released by αMMH. The testing of this compound and the principles behind the long term aims of its study did, however, lead to the development of the study of CO-RMs in this Thesis.

11.4 Appendix 4: Step by Step Protocol for the Myoglobin Assay

The following section comprises a step by step guide to performing a myoglobin assay to test the CO release from CO-RMs.

1) Prepare the spectrophotometer making sure the cuvettes (1.5 ml plastic) (Sarstedt) are in place and that they are at 37 °C (heated carousel).
2) Zero the baseline using 1 ml PBS (0.01M, pH = 7.4) (Sigma)
3) Prepare a 66 µmol/l (final concentration) stock of myoglobin (lysophilised horse heart) (Sigma) by adding 50 ml PBS (0.01M, pH = 7.4) (Sigma) to 56 mg myoglobin.
4) Warm the myoglobin in a water bath at 37 °C.
5) Once warm add sodium dithionite (0.1 % or ~ 2 heaped spatulas) (Sigma) to convert the myoglobin stock to deoxymyoglobin (deoxy-Mb).
6) Add 1 ml deoxy-Mb to a cuvette and read the absorbance between 500 and 600 nm. This should be a single peak around 560 nm. This is your zero MbCO value.
7) With the same cuvette bubble the deoxy-Mb with CO gas for 1 min to saturate the myoglobin. Read the sample again. The curve should now have two peaks with the second peak smaller than the first. The first peak should be around 540 nm and the second 578 nm. This is your 100 % MbCO value.
8) Using the deoxy-Mb stock add 1 ml to a cuvette.
9) Prepare the CO-RM you wish to test. The final concentrations used in this study were 20, 40 and 60 µM. These were prepared as 4, 8 and 12 mM stocks respectively. The final volume added, therefore, was 5 µl.
10) Add your compound to the myoglobin.

11) Mix the myoglobin-CO-RM solution gently using a Gilson pipette (1000 µl) so as not to oxygenate the solution. This would result in two peaks of equal size.

12) Layer the myoglobin with 500 µl of mineral oil (Sigma) to prevent CO escaping and the myoglobin becoming oxygenated.

13) Read the sample. For the purpose of this study samples were read every 5 min for the first 30 min and every 30 min thereafter. Depending on the CO-RM there will either be no change, a gradual conversion from deoxy-Mb to MbCO or a near instant conversion.

14) Using the readings obtained and the calculations specified in paragraph 2.4 it is then possible to determine the concentration of MbCO and, therefore, the CO release.

11.5 Appendix 5: Full Publications by the Author

Presented in this section are the full articles which I have written or contributed to during my PhD. The papers are presented in chronological order and are set out as published with the exception of the most recent paper "Bioactive properties of iron-containing carbon monoxide-releasing molecules (CO-RMs)" in the JPET which is currently only available as an Epub ahead of print.

FEBS 25506

FEBS Letters 508 (2001) 403 406

Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia

Philip Sawle, Roberta Foresti, Colin J. Green, Roberto Motterlini*

PII: S0014-5793(01)03117-9

The FASEB Journal express article10.1096/fj.04-2169fje. Published online November 19, 2004.

CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule

Roberto Motterlini,* Philip Sawle,* Sandip Bains,* Jehad Hammad,* Roger Alberto,† Roberta Foresti,* and Colin J. Green*

British Journal of Pharmacology (2005) 145, 800–810
www.nature.com/bjp

Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages

[1]Philip Sawle, [1]Roberta Foresti, [2]Brian E. Mann, [2]Tony R. Johnson, [1]Colin J. Green & *,[1]Roberto Motterlini

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Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 16 (2006) 995–998

η^4-Pyrone iron(0)carbonyl complexes as effective CO-releasing molecules (CO-RMs)

Ian J. S. Fairlamb,[a,*] Anne-Kathrin Duhme-Klair,[a] Jason M. Lynam,[a] Benjamin E. Moulton,[a] Ciara T. O'Brien,[a] Philip Sawle,[b] Jehad Hammad[b] and Roberto Motterlini[b,*]

0960-894X/$ - see front matter
doi:10.1016/j.bmcl.2005.10.085

JPET Fast Forward. Published on April 7, 2006 as DOI:10.1124/jpet.106.101758

JPET #101758

BIOACTIVE PROPERTIES OF IRON-CONTAINING CARBON MONOXIDE-RELEASING MOLECULES (CO-RMs)

Philip Sawle[1], Jehad Hammad[1], Ian J. S. Fairlamb[2], Benjamin Moulton[2], Ciara T. O'Brien[2], Jason M. Lynam[2], Anne K. Duhme-Klair[2], Roberta Foresti[1] and Roberto Motterlini[1]

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